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THE BIOCHEMISTRY OF ANAEROBIC DIGESTION

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The degradation of propionic, succinic and lactic acids appear to be closely related with common intermediates. Propionic acid can be degraded through lactic or succinic acids and the reverse is true for each of the other two compounds. ~~In addition,~~ propionic acid may also be metabolized by alpha or beta-oxidation giving rise to an intact methyl group in the methane formed, from either the number two or three carbon. A pathway for glutamic acid utilization was postulated involving the formation of two moles of acetic acid for each mole of substrate used. Oleic acid was primarily degraded by beta-oxidation. In the fermentation of benzoic acid, three moles of acetic acid were produced per mole degraded and the number one carbon was primarily converted to methane.

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THE BIOCHEMISTRY OF ANAEROBIC DIGESTION

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Federal Water Quality Administration
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THE BIOCHEMISTRY OF ANAEROBIC DIGESTION

SUMMARY

The major emphasis in this investigation was to determine biochemical mechanisms in the anaerobic degradation of typical organics found in wastes by means of radioactive tracer techniques.

Two liter laboratory scale digesters were used in all the radioactive tracer work. Substrate and nutrients were slug fed daily while carbon-14 compounds were slug fed one time or daily depending on the type of study being performed. Chemical analyses of the liquid feed and effluent were routinely performed along with identification of gaseous end products. Radioactivity analyses on the gases produced were conducted using liquid scintillation techniques.

The degradation of propionic, succinic and lactic acids appear to be closely related with common intermediates. Propionic acid can be degraded through lactic or succinic acids and the reverse is true for each of the other two compounds. In addition, propionic acid may be metabolized by alpha or beta-oxidation giving rise to an intact methyl group in the methane formed, from either the number two or three carbon. A pathway for glutamic acid utilization was postulated involving the formation of two moles of acetic acid for each mole of substrate used. Oleic acid was primarily degraded by beta-oxidation. In the fermentation of benzoic acid, three moles of acetic acid were produced per mole degraded and the number one carbon was primarily converted to methane.

Other studies receiving support from this research grant included the development of a rapid COD procedure which was employed during the course of the investigation. The procedure saves significant time, is less costly and gives excellent results. Also, it was determined, using eight-liter digesters fed once daily, that ammonia-nitrogen was the best form of nutrient nitrogen for anaerobic treatment systems. Further, in the utilization of glucose in batch fed biological treatment systems, zero order kinetics of glucose removal was obtained.

I. INTRODUCTION

Anaerobic processes are important in waste treatment for the removal of concentrated biodegradable matter. Present methods of process design which incorporate anaerobic techniques are, for the most part, empirical and probably do not function as effectively as possible. To a large degree this is because of a lack of information on the biological mechanisms and reactions which occur in the anaerobic digestion process. The major purpose of this study was to determine some of the metabolic pathways involved in the digestion of organic compounds which are constituents of complex wastes and thus add data to the literature concerned with anaerobic treatment.

Anaerobic digestion processes are used in municipal waste treatment to dispose of the sludges produced from settling raw sewage and aerobic process effluents. Aerobic processes and settling remove solids and oxygen-consuming material from the waste stream by producing sludge. This sludge, however, must be further treated before disposal to reduce its volume and to remove its objectionable properties. The material balance of a municipal sewage treatment plant shows that the major portion of the influent organic waste load is actually treated in the digester. The end products of anaerobic digestion are gas (methane and carbon dioxide) and a more concentrated, less obnoxious sludge.

Recently, several processes using anaerobic treatment methods have been developed to handle difficult and unusual wastes. The anaerobic contact process (anaerobic activated sludge) has been successfully applied to the treatment of packing house wastes. Laboratory scale tests on the anaerobic filter have demonstrated the potential of this technique. These and most other treatment processes are designed to take advantage of the high organic loadings which can be handled by anaerobes.

At the present time, anaerobic units are designed empirically with little or no understanding of process requirements and mechanisms. This lack of sufficient theoretical analysis has been common in the history of science and technology; however, to achieve optimal large scale application and utility of any technique, available background information should be utilized and theory should be amply developed. Therefore, if anaerobic treatment is to be used to its utmost potential, a detailed knowledge of the process is a necessary prerequisite.

A fundamental understanding of anaerobic processes is lacking. Only peripheral knowledge is available today. Information on the basic biochemistry of the process has been scattered throughout the biological, chemical and sanitary engineering literature. There is little question that a familiarity with the basics of the process will help engineers to properly design and operate anaerobic treatment units.

Generally the anaerobic treatment process has been considered as a two component biological system. The first includes the group of microorganisms called the non-methane formers and second, the methane formers. Often the former are referred to as "the acid producers".

The non-methane formers are composed of facultative and anaerobic organisms whose prime function is the conversion of complex organic compounds, including proteins, carbohydrates, and fats, to relatively simple organics. A few of the many end-products produced include such compounds as acetic, propionic and lactic acids, ethanol, and carbon dioxide.

The second group are very specialized and unique in their ability to produce methane gas. They are very strict anaerobes with a slow rate of growth, both factors probably contributing to the difficulty experienced in their isolation and culture. Their uniqueness is further characterized by their ability to utilize only simple organics which fortunately are comprised mainly of the end products of the non-methane forming group.

Knowledge of the biochemistry involved in methane fermentation has grown very slowly through the years and the process is still not clearly understood. Much research in this area has been directed toward the study of the microbiology of the methane formers, but under conditions which are non-existent in an actual anaerobic digester. The purpose of this investigation was to simulate field anaerobic digestion in laboratory units and to study the biochemical mechanisms of the predominate bacterial flora.

Some attempts to understand the biochemistry of the methane fermentation using mixed cultures have been made. The early work in sewage sludge digestion was limited to the identification of the volatile acids present, the quantities of methane and carbon dioxide formed and the reduction of sludge volumes. Some work was conducted using pure compounds, including fatty acids, carbohydrates, alcohols, proteins and one-carbon compounds. With the exception of fatty acids, alcohols and one-carbon compounds little has been done to define the degradative biochemical mechanisms.

In the present investigation a series of laboratory digesters were fed on a batch basis resembling the typical operation of field installations. The organic substrates used included: acetic acid, propionic acid, butyric acid, oleic acid, lactic acid, pyruvic acid, succinic acid, glutamic acid, benzoic acid, glucose and others. Inorganic salts were fed in sufficient concentrations to allow unrestricted growth. Routine analyses to determine the sludge pH, alkalinity, volatile acids, COD, and gas composition were conducted to control the operation of the digesters.

Various radioactive labeled organic compounds were fed to the digesters for either long or short periods of time. The gases produced were then analyzed to determine their composition and radioactivity. These data were used to confirm postulated metabolic pathways for the particular compound under study. Anaerobic Warburg studies were conducted to provide independent confirmation of these results.

This project, then, hopes to provide needed information towards the understanding and efficient utilization of anaerobic decomposition processes. It is hoped that the present work in conjunction with other studies in this field will provide the theoretical basis for improved anaerobic waste treatment operation and design.

In addition to the studies in biochemistry, several additional and significant investigations receiving support from the research grant were conducted. These studies included the effects of various forms of nitrogen used as nutrients in anaerobic digestion, the kinetics of glucose disappearance in biological treatment processes and a rapid method COD test, which was used during the conduct of the work. This work is presented as Sections I, II and III of the Appendix.

II. LITERATURE SURVEY

Methane fermentation is a phenomenon recognized since early times and understood as the cause of the faint glow occurring in swamps. The biochemistry of its formation, however, has remained a mystery until recent times. The reason for this has been the inability to maintain the methane forming organisms in pure cultures. Much of the past work has involved the study and identification of the intermediates and end products in the anaerobic digestion of sewage sludges.

The literature survey will be divided into two parts. One part will deal with the formation of methane from known sources. The second part will discuss related non-methane fermentations.

Heukelekian and Heinemann (1) reported that the methane producing organisms are commonly believed to be unable to metabolize complex sewage organics such as proteins, fats and cellulose. These organics must first be hydrolyzed into simpler compounds such as acetate, butyrate and ethanol before methane formers can utilize them. Therefore, other bacteria must liquify the complex organics in fresh sewage sludge.

A. Fatty Acids and One-Carbon Compounds

The common facultative and anaerobic non-methane producing organisms cannot utilize fatty acids under the strict anaerobic environment of the digester. Under such conditions these organisms need the proper hydrogen acceptors such as oxygen, nitrates or sulfates for oxidation. Therefore, degradation can only be carried out by a particular group of organisms, which have a unique hydrogen acceptor system. These organisms are known as the methane formers.

1. Acetate and One-Carbon Compounds

Buswell and Neave (2) reported that in 1887 Hoppe-Seyler found that mixed cultures degraded acetate, producing the same volume of methane and carbon dioxide, and they verified this early result in their own laboratory.

Pine and Barker (3) used deuterium labelled acetate to determine if the hydrogen in the methyl group of acetate was removed by an oxidative reaction or if it remained intact. They found that the methyl group was transferred intact to methane according to the following equation:

$$CD_3COOH \longrightarrow CD_3H + CO_2$$

Using acetate with deuterated water they found that one atom of deuterium per molecule of methane was derived from the water. The experiment may be expressed by the following equation:

$$CH_3COOH \xrightarrow{D_2O} CH_3D + CO_2$$

From this work the following general metabolic scheme was suggested:

$$\begin{tikzcd}
CH_3COOH + HX \arrow[r, "-2H"] & CH_3X + CO_2 \arrow[d, "+2H"] \\
& CH_4 + HX
\end{tikzcd}$$

In this reaction, HX would be an unidentified organic compound.

Barker (4) indicated that a Methanosarcina rapidly degraded methanol according to the following equation:

$$4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$$

Stadtman and Barker (5) used C^{14} labelled carbon dioxide to test the above predictions in 1951. Since the results showed that from 98 to 99 percent of the methane was derived from the methanol molecule, the methane must be formed directly from the reduction of the methanol. The reactions can be expressed by the following equations:

$$(1)\quad CH_3OH + H_2O \longrightarrow CO_2 + 6H$$

$$(2)\quad 3CH_3OH + 6H \longrightarrow 3CH_4 + 3H_2O$$

$$\text{Net:}\quad 4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$$

It was concluded, therefore, that methanol was a source of methane.

Stephenson and Strickland (6) isolated an organism which could use formate as a sole carbon source according to a two stage process expressed by the following equations:

$$HCOOH \longrightarrow H_2 + CO_2$$

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$$

The reactions involved two enzymes, a hydrogenase and a hydrogenlyase. The organism which was tested in a cell suspension reduced carbon monoxide, carbon dioxide, formate, methanol and formaldehyde to methane in the presence of hydrogen.

Barker (4) reviewed Kluyver and Schnellen's (7) work on the fermentation of one-carbon compounds by pure cultures of three species of methane producing organisms in the presence of hydrogen. Methanobacterium formicicum which was incapable of reducing methanol or formaldehyde but was able to ferment formate was found to convert carbon dioxide to methane in the presence of hydrogen. This organism also reduced carbon monoxide, but only at low concentrations of carbon monoxide. Methanosarcina barkerii could ferment acetate and methanol but could not reduce formate or formaldehyde. This organism also reduced carbon monoxide to methane. Since carbon dioxide was produced during the carbon monoxide fermentation, it was believed that carbon dioxide was an intermediate in the carbon monoxide reduction to methane. Methanobacterium omelianskii was found to be incapable of using carbon monoxide or any other one-carbon compound except carbon dioxide for reduction to methane.

Using what was believed to be a pure culture of Methanobacterium omelianskii, Pine (8) studied the formate fermentation with a deuterium label and a formic dehydrogenase inhibitor. It was reported that formate was principally converted to carbon dioxide before reduction to methane. When external carbon dioxide was present with or without the inhibitor, very little of the formate, if any, was reduced to methane directly. Recent studies by Bryant, Wolin, Wolfe, and Mandel (9) showed that Methanobacterium omelianskii cultures actually contained two species of bacteria and was not a pure culture. One species produces H_2 from ethyl alcohol but is inhibited by H_2. The other uses H_2 to reduce carbon dioxide to methane.

In the course of this literature survey three verified sources of methane were identified; a fourth speculative source was also discussed.

These sources are listed as follows:

KNOWN SOURCES OF METHANE
Carbon dioxide reduction
Methanol
Acetate
SPECULATIVE SOURCES OF METHANE
Formate

2. Fatty Acids

Buswell and Neave (2) in 1930 suggested the following general formula which was applicable to the conversion of saturated fatty acids to carbon dioxide and methane:

$$C_nH_{2n}O_2 + (\frac{n-2}{2})H_2O \longrightarrow (\frac{n+2}{4})CO_2 + (\frac{3n-2}{4})CH_4$$

Since hydrogen gas was never found in this reaction, they concluded that Knoop's beta-oxidation theory could not be applied to these short length fatty acids.

For the long chain fatty acids, a modification of Knoop's beta-oxidation scheme was proposed as follows:

(1) $RCH_2\text{-}CH_2\text{-}COOH \longrightarrow RCH\text{-}CH\text{-}COOH+H_2$
(2) $RCH{=}CH\text{-}COOH+H_2O \longrightarrow RCH(OH)\text{-}CH_2\text{-}COOH$
(3) $RC(OH)_2\text{-}CH_2\text{-}COOH \longrightarrow R\text{-}COOH+CH_3\text{-}COOH$

The hydrogen acceptors suggested were:

1. Microorganisms in the sludge which used the hydrogen.
2. Carbon dioxide which reacted with hydrogen to form methane.
3. Carbohydrates or proteins which were being decomposed at the same rate.

In 1934, Tarvin and Buswell (10) determined some of the intermediates produced from a number of organic compounds. The substrates studied were slug fed in large amounts to the digesters. As the fermentation was progressing, samples were taken and analyzed. The following list shows some of the substrates studied and the results:

Substrate	Product of Fermentation
Formate	-
Ethanol	Acetate, propionate, acetaldehyde
Propionate	Acetate, formate
Isobutyrate	Lower fatty acids
Lactate	Propionate
Succinate	Propionate
Pyruvate	Acetate, formate, acetaldehyde
Glucose	Formate, acetate, propionate, butyrate, lactate

At the conclusion of this work they indicated that alpha-oxidation was probably involved in the degradation of fatty acids rather than beta-oxidation. Further experiments using Knoop's method of feeding phenyl substituted fatty acids did not prove conclusively that alpha-oxidation was involved.

Buswell and Hatfield (11) reported that propionate was produced when an acetate acclimated digester was fed lactic acid. It was found that the acetate originally present disappeared first and was then followed by propionate. The use of propionate as the substrate resulted in no buildup of any other acid.

In a study of the methane fermentation of fatty acids, Stadtman and Barker (33) concluded that (1) the carboxyl carbon of propionate was converted to carbon dioxide, (2) the methane produced when acetate was an end product, came mostly from carbon dioxide reduction, and (3) valerate was fermented by means of beta-oxidation, and butyrate and caproate were most likely fermented by the same mechanism.

Using propionic acid, labeled on the number, 1, 2, and 3 carbons, and enrichment cultures, Buswell et al (34) reported that all three carbons of propionate could give rise to carbon dioxide and methane. These results were contradictory to those of Stadtman and Barker (5).

In the metabolism of propionic acid by the mitochondria of animal tissues as reported by Flavin, et al (12) and Lardy and Adler (13) results indicated that carbon dioxide was taken up and that propionate was oxidized to succinate. This type of process could quite possibly occur in the presence of anaerobic sludge. The succinate found could be oxidized via the tricarboxylic acid cycle.

A likely end product could be acetic acid which can be degraded by methane bacteria producing carbon dioxide and methane. Also, Phares et al (14) reported that cell extracts of Propionibacterium pentosaceum carboxylate propionate rapidly in the presence of CoA and succinate but not in their absence.

The reverse reaction has also been repeatedly demonstrated in Propionibacteria by Delwiche et al (15) and Wood et al (16). In this case succinate is converted to propionate and carbon dioxide. Direct reversibility of succinic acid with propionic acid and carbon dioxide was implied by the studies of Barbon and Ajl (36) using Propionibacterium pentosaceum. It is possible that this interchangeability is also a characteristic of the processes occurring in anaerobic digestion.

Gutierrez (37) found that lactic acid formed propionic acid using some anaerobic gram-negative cocci existing in the rumen of cattle and other animals. It is also known that propionic acid can be formed from lactic acid by Clostridium propionicum by a different route from that in the Propionibacteria.

In cheese, the main substrate is lactic acid, and it was this fermentation that was first studied. The general equation given by Fitz (38) is approximately:

$$3CH_3CHOHCOOH \longrightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$$

Von Freudenreich and Orla-Jensen (39) found with pure cultures, however, that the ratio of propionate to acetate was not quite 2, but 1.6 to 1.8; Van Niel (40) generally obtained similar results, but found the ratio of acetate to carbon dioxide to be 1.

This consistancy of the latter ratio suggested that the acetic acid and carbon dioxide must arise from the same precursor, which would be pyruvate according to the following anaerobic decarboxylation reaction:

$$CH_3COCOOH + H_2O \longrightarrow CH_3COOH + CO_2 + 2H \quad (1)$$

Since the conditions are anaerobic, a compound other than oxygen must act as the hydrogen acceptor. But the formation of pyruvic acid from lactic acid is itself a dehydrogenation:

$$CH_3CHOHCOOH \longrightarrow CH_3COCOOH + 2H \quad (2)$$

Thus for every mole of acetic acid formed there are four hydrogens to be accepted. If the pure culture were Clostridium propionicum these four hydrogen atoms produced in the conversion of lactate to pyruvate and of pyruvate to acetate, Equations (1) and (2), would be directly transferred to lactate, reducing it to propionate:

$$2CH_3CHOHCOOH + 4H \longrightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

Thus, the equation of Fitz would be merely the sum of reactions (1), (2), and (3).

Proof that in these organisms propionic acid arises by direct reduction of a 3-carbon precursor is given by a C^{14} experiment by Leaver et al (35). Lactate with C^{14} in the number three carbon (C-3) (methyl) position gave rise to propionate labeled only in the C-3 position:

$$\overset{*}{C}H_3CHOHCOOH \xrightarrow{-H_2O} \overset{*}{C}H_2{=}CHCOOH \xrightarrow{+2H} \overset{*}{C}H_3CH_2COOH$$

By contrast, with propionibacteria both the C-3 and the C-2 positions of the propionate contained the isotope. The same was true using pyruvate or lactate labeled in the C-2 position. Evidently, therefore, the C-3 and C-2 carbons of propionate become equivalent at some stage in the process, and this could mean that succinate or a similar compound could be an intermediate. Since it was found that several propionibacteria could form propionate by decarboxylating succinate as previously indicated Delwiche, (15) and also Johns (41), an indirect route via the four-carbon acid was possible. Figure 1 gives a possible pathway.

Jeris and McCarty (17) using octanoic and palmitic acids as substrates in 1962 suggested a theory for the degradation of medium and long chain fatty acids. They reported, upon using octanoic acid-1-C^{14}, that either beta or omega-oxidation was possible but, with octanoic acid-7-C^{14} labeled, however, omega-oxidation could be ruled out as an important mechanism. Therefore, they concluded that octanoic acid was primarily degraded by beta-oxidation. The reactions proposed in the beta-oxidation of octanoic acid are shown in Figure 1A.

In the study of palmitic acid, Jeris and McCarty (17) found that using palmitic acid-6-C^{14}, the results showed that the number six carbon was converted primarily to methane, indicating that beta or omega-oxidation were possible mechanisms while with the number eleven carbon labeled, it was found that beta-oxidation was the prime mechanism involved and omega-oxidation played a secondary role, if any at all. From these results, they concluded that beta-oxidation was the primary mechanism involved in the degradation of medium and long-chain saturated fatty acids.

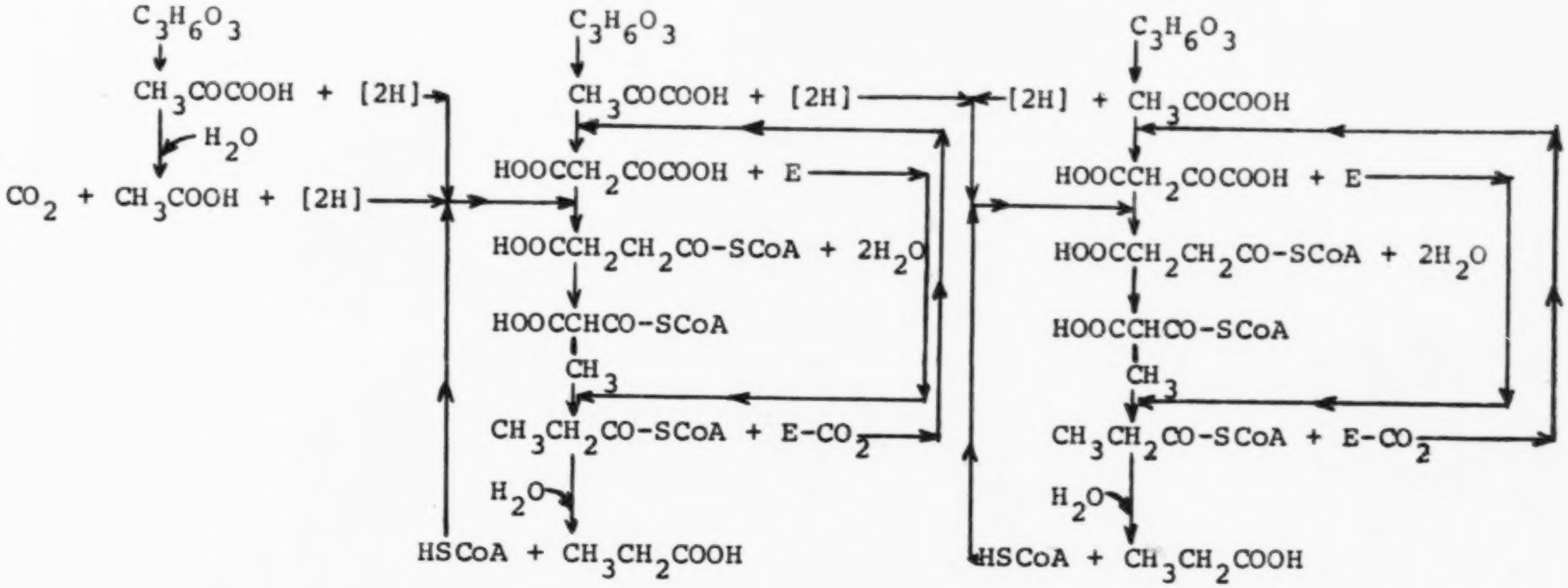
B. Proteins

In general the breakdown of proteins proceeds by an attack on the peptide linkage which forms a series of polypeptides. Further breakdown yields amino acids which may eventually enter the tricarboxylic acid cycle as hydroxy or keto-acids.

In a review of the metabolism of amino acids Fruton and Simmonds (18) indicated that deamination may occur by oxidation, reduction and transamination. Gortner and Gortner (19) claimed that both anaerobes and aerobes possess deamination mechanisms. With anaerobes reductive deamination produces a saturated fatty acid (Eq. 1), while desaturative

FIGURE 1

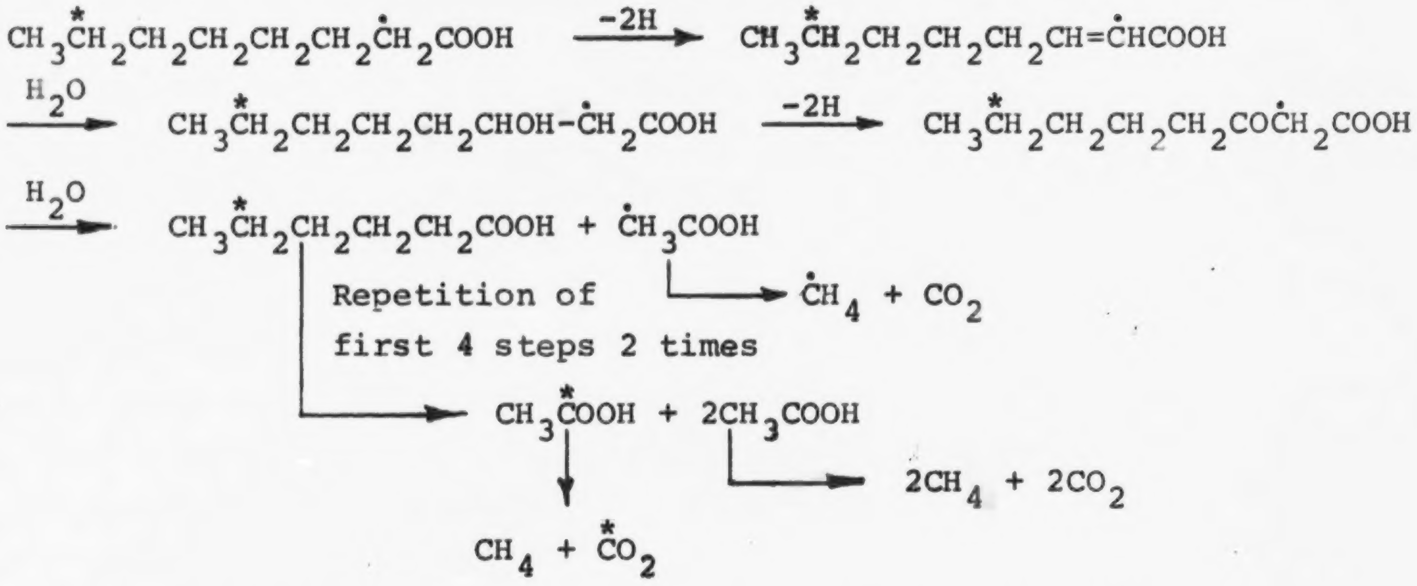
POSSIBLE PATHWAY FOR LACTIC ACID



NET REACTION: $3CH_3CHOHCOOH \longrightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$

FIGURE 1A

BETA-OXIDATION OF OCTANOIC ACID


$CH_3\overset{*}{C}H_2CH_2CH_2CH_2CH_2\dot{C}H_2COOH \xrightarrow{-2H} CH_3\overset{*}{C}H_2CH_2CH_2CH_2CH{=}\dot{C}HCOOH$
$\xrightarrow{H_2O} CH_3\overset{*}{C}H_2CH_2CH_2CH_2CHOH{-}\dot{C}H_2COOH \xrightarrow{-2H} CH_3\overset{*}{C}H_2CH_2CH_2CH_2CO\dot{C}H_2COOH$
$\xrightarrow{H_2O} CH_3\overset{*}{C}H_2CH_2CH_2CH_2COOH + \dot{C}H_3COOH$
$\dot{C}H_4 + CO_2$
Repetition of first 4 steps 2 times
$CH_3\overset{*}{C}OOH + 2CH_3COOH$
$2CH_4 + 2CO_2$
$CH_4 + \overset{*}{C}O_2$

(1) $CH_3\overset{*}{C}H_2(CH_2)_5\ COOH + 6H_2O \longrightarrow 4CH_4 + 3CO_2 + \overset{*}{C}O_2 + 12H$

(2) $12H + 3/2\overset{\circ}{C}O_2 \longrightarrow 3/2\overset{\circ}{C}H_4 + 3H_2O$

Net: $CH_3\overset{*}{C}H_2(CH_2)_5COOH + 3H_2O \longrightarrow 3/2\overset{\circ}{C}H_4 + 4CH_4 + 5/2CO_2$

Using $NaHC^{14}O_3$ or odd-numbered carbon-14 the specific activity (SA) is:

$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles radioactive } CH_4} = \frac{3/2 + 4}{3/2} = 3.67$$

* and · indicate C^{14}

° Indicates reduced radioactivity due to dilution with CO_2.

deamination produces an unsaturated fatty acid (Eq. 2). In addition, transamination (Eq. 3) and decarboxylation (Eq. 4) reactions can occur.

$$(1)\quad RCH(NH_2)COOH + H_2 \longrightarrow RCH_2COOH + NH_3$$

$$(2)\quad RCH_2CH(NH_2)COOH \longrightarrow RCH{=}CHCOOH + NH_3$$

$$(3)\quad RCH_2COCOOH + R'CH(NH_2)COOH \rightleftharpoons RCH_2CH(NH_2)COOH + R'CHCOCOOH$$

$$(4)\quad RCH(NH_2)COOH \longrightarrow RCH_2NH_2 + CO_2$$

McKinney (20) stated "The anaerobic degradation of proteins results in the formation of amino acids which are hydrolytically deaminated to form the equivalent hydroxy acid". This is illustrated in the following reactions:

$$\text{Protein} \longrightarrow 2NH_2\overset{R}{C}HCOOH \xrightleftharpoons[+H_2O]{-NH_3} 2\overset{R}{C}HOHCOOH$$

The following formula was developed by Symons and Buswell (21) for predicting the fermentation results for complex organic wastes including proteins:

$$C_nH_aO_bN_cS_dM_{ev} + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{7c}{4} + \frac{d}{2} + \frac{3ev}{4}\right)H_2O$$

$$\longrightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} - \frac{5c}{8} + \frac{d}{4} - \frac{9ev}{8}\right)CO_2$$

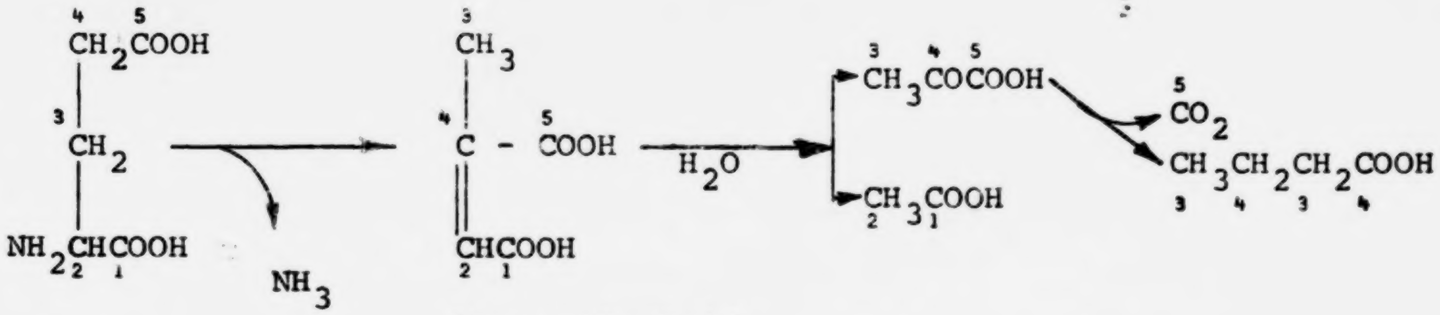
$$+\left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8} - \frac{d}{4} + \frac{ev}{8}\right)CH_4 + cNH_4HCO_3$$

$$+ dH_2S + eM(HCO_3)v$$

Where: M = Metal ion
v = Valence of M

1. Glutamic Acid

In 1956, Wachsman and Barker (22) using the anaerobe *Clostridium tetanomorphum*, reported that the fermentation of glutamic acid to NH_3, CO_2, acetic acid, and butyric acid involved the intermediate formation of 2-methylfumaric acid (mesaconic acid). From studies with C^{14} labeled substrates they showed that the fermentation was accompanied by the transformations shown in the following scheme:



Mesaconic Acid

The degradation of glutamic acid in most biological systems proceeds by the oxidation of α-ketoglutaric acid in the citric acid cycle.

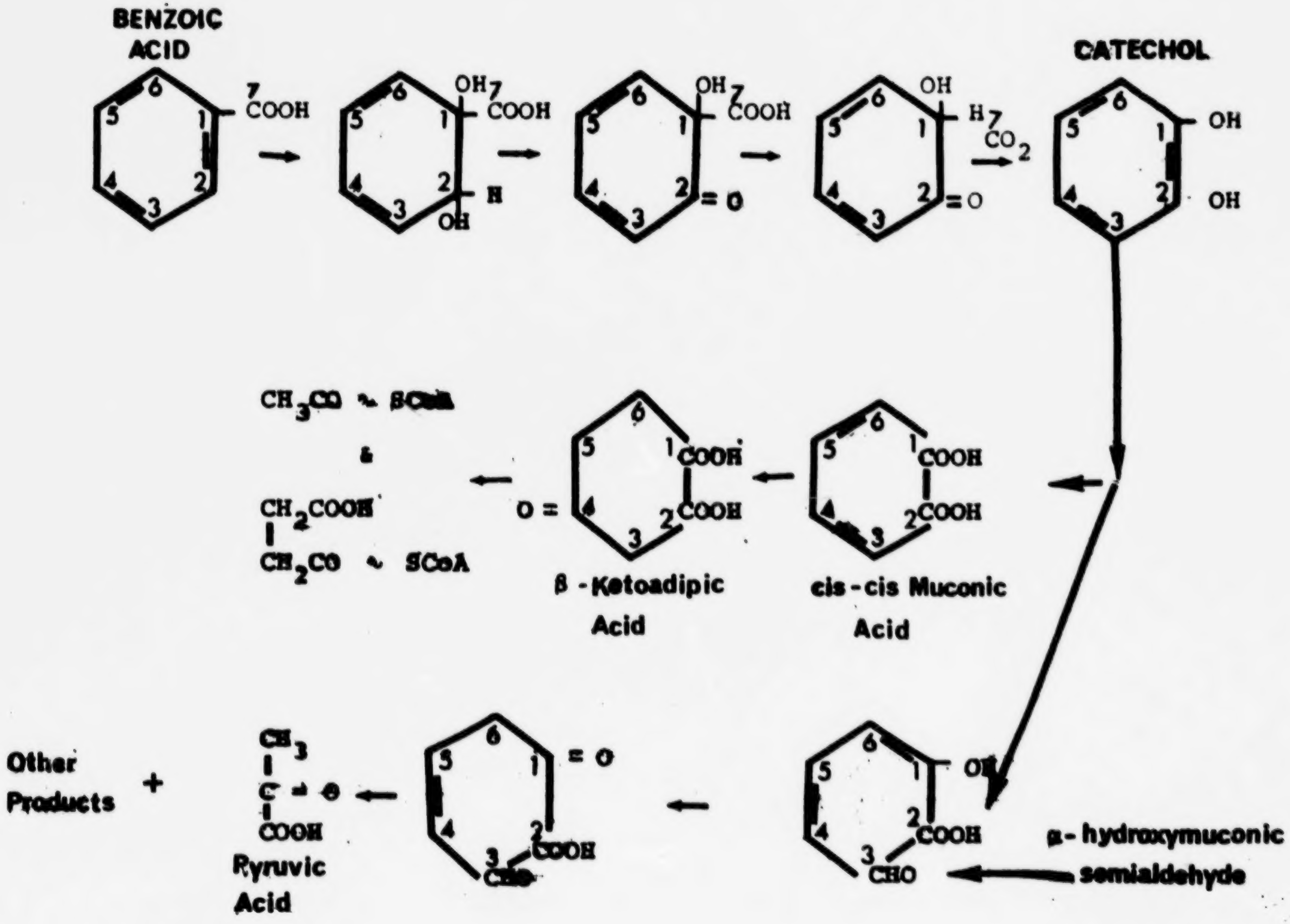
C. Benzoic Acid

The modus operandi for the metabolism of benzoic acid by methane bacteria and other anaerobes has not been determined. Several aerobic pathways are known however, and it is possible that similar pathways may be employed anaerobically; several of these pathways will be discussed.

Benzoic acid is known to be oxidized to catechol along with several other aromatic compounds. The initial reaction in the conversion to the catechol is decarboxylation; thus, if benzoic acid is labeled in the #7 carbon position, the catechol produced is unlabeled (23, 24). The key intermediate in the benzoic acid degradation is catechol. Two pathways found in bacteria for the cleavage of the benzene ring are known. Following the formation of catechol the ring may be split either between carbon #1 and #2 or adjacent to one of the hydroxyl groups (18, 25). When split between the hydroxyl groups, cis-cis muconic acid is formed, then β-ketoadipic acid and finally succinyl and acetyl CoA. With a pseudomonas, α-hydroxy-muconic semi-aldehyde is formed followed by pyruvate and other end products. The reactions are illustrated in Figure 2.

Some of these reactions may take place in the presence of facultative organisms in digester sludge. The succinate and pyruvate formed may be converted to acetate by the tricarboxylic acid cycle. The acetate would then be utilized by the methane formers present.

FIGURE 2
DEGRADATION OF BENZOIC ACID



III. THEORETICAL CONSIDERATIONS

Organic compounds labeled in a known manner, are very often used to determine biochemical mechanisms. The resultant end products of the biological degradation are marked with distributions of radioactivity and may be assayed. The conclusions are drawn from these distributions in the end products.

It is very fortunate that the majority of the organic compounds metabolized in anaerobic treatment yield the gaseous end products, carbon dioxide and methane. These gaseous products are easily collected and separated and the radioactivity of each can be determined without requiring any of the cumbersome analyses which are often required with non-gaseous end products.

A. Sources of Methane

Since methane is one of the major end products, its sources are very important. In the review of the literature three known sources were described and are illustrated again as follows:

1. Carbon dioxide reduction

$$\overset{*}{C}O_2 + 8H \longrightarrow \overset{*}{C}H_4 + 2H_2O$$

2. Methanol

$$4\overset{*}{C}H_3OH \longrightarrow 3\overset{*}{C}H_4 + \overset{*}{C}O_2 + 2H_2O$$

3. Acetate

$$\overset{*}{C}H_3COOH \longrightarrow \overset{*}{C}H_4 + CO_2$$

A fourth speculative source of methane was formate. This reaction may be expressed by the following equation:

4. Formate

$$4HCOOH \longrightarrow CH_4 + 3CO_2 + 2H_2O$$

Evidence against the direct reduction of formate to methane is too strong to consider formate as a source of methane in this research.

B. Sodium Bicarbonate or Carbon Dioxide

Labeled C^{14} sodium bicarbonate can be used to determine the amount of carbon dioxide which is reduced to methane. As bicarbonate and soluble carbon dioxide are rapidly interconvertible, reference will be made to carbon dioxide only for simplicity. The reaction involved in carbon dioxide reduction may be expressed by the following equation:

$$\underset{(NaH\overset{*}{C}O_3)}{\overset{*}{C}O_2} + 8H \longrightarrow CH_4 + 2H_2O$$

The distribution of radioactivity in the methane and carbon dioxide can then be determined. When using labeled carbon dioxide the only labeled methane must come from carbon dioxide reduction. All other methane produced must come from acetate since this is the only other source of methane under the given experimental conditions.

As an illustration of the principles involved, the specific activity of carbon dioxide and methane produced from the methane fermentation of a certain substrate in the presence of C^{14} labeled carbon dioxide will be assumed at 200 and 50 respectively. Since the methane has only one-fourth the specific activity of the carbon dioxide, three moles of non-radioactive methane must have been formed for each mole of radioactive methane. The specific activity ratio of carbon dioxide to methane may then be expressed in terms of methane alone as follows:

$$\text{Specific Activity (SA)} \ \frac{CO_2}{CH_4} = \frac{200}{50} = 4$$

$$\text{or SA} \ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4 \text{ produced}}{\text{Moles of Radioactive } CH_4 \text{ or moles of } CO_2 \text{ reduced to } CH_4} = \frac{4}{1} = 4$$

Since three moles of non-radioactive methane was formed for every four moles of methane, it can be concluded that 75 percent of the methane came from acetic acid since this substance was the only other source of methane.

C. Propionic Acid

Figures 3 and 4 have been prepared to illustrate two possible metabolic pathways for propionic acid in anaerobic digestion. In the reactions shown in Figure 3 each mole of propionic acid is hydrolyzed to yield one mole of acetic acid, one mole of formic acid and four hydrogen atoms. The acetic acid is further broken down to methane and carbon dioxide while the formic acid yields carbon dioxide and two more hydrogen atoms. At this point a total of six hydrogen atoms are formed from each mole of propionic acid and made available for the reduction of carbon dioxide.

From the reactions shown in Figure 3, carbons numbered one and two will form carbon dioxide and part of the carbon dioxide will be reduced to methane, while carbon number three will form methane only.

When C^{14} carbon dioxide is used or is formed during the degradation of propionic acid, a portion of the radioactive carbon dioxide will be reduced to radioactive methane. The specific activity (S.A.) ratio of carbon dioxide to methane based on the mechanism shown in Figure 3 may be computed as follows:

$$\text{S.A.}\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles of } CO_2 \text{ reduced}} = \frac{7/4}{3/4} = 2.33$$

Another possible pathway is given in Figure 4 in which propionic acid is carboxylated at the omega position forming succinic acid as previously mentioned in the literature survey. Further reaction of the symmetrical succinate may occur via the tricarboxylic acid cycle and eventually acetate and carbon dioxide may be formed. The acetate produced may then be degraded by methane organisms in the usual manner to yield carbon dioxide and methane. By proceeding through succinate, a label on either the number 2 or 3 carbon becomes randomized among these two carbon atoms. Thus, the results obtained are different than those expected with direct decarboxylation.

Inspection of Figure 4 shows that if the number 2 or 3 carbon is labeled it may become either radioactive carbon dioxide or radioactive methane. Therefore, the label becomes randomized between the methane and carbon dioxide. From each mole of propionic acid utilized, one-half mole of radioactive methane and carbon dioxide is initially produced. A portion of the radioactive carbon dioxide will then be reduced to methane and amounts to 3/16 of a mole. Following

FIGURE 3

POSSIBLE PATHWAY FOR PROPIONIC ACID

(1) $\overset{3}{C}H_3\overset{2}{C}H_2\overset{1}{C}OOH + 2H_2O \longrightarrow \overset{3}{C}H_3\overset{2}{C}OOH + H\overset{1}{C}OOH + 4H$

(2) $\overset{3}{C}H_3\overset{2}{C}OOH \longrightarrow \overset{3}{C}H_4 + \overset{2}{C}O_2$

(3) $H\overset{1}{C}OOH \longrightarrow \overset{1}{C}O_2 + 2H$

(4) $6H + 3/4CO_2 \longrightarrow 3/4CH_4 + 3/2H_2O$

Net: $CH_3CH_2COOH + 1/2H_2O \longrightarrow 5/4CO_2 + 7/4CH_4$

If $NaHC^{14}O_3$ or HPr-1 or 2C-14 is used:

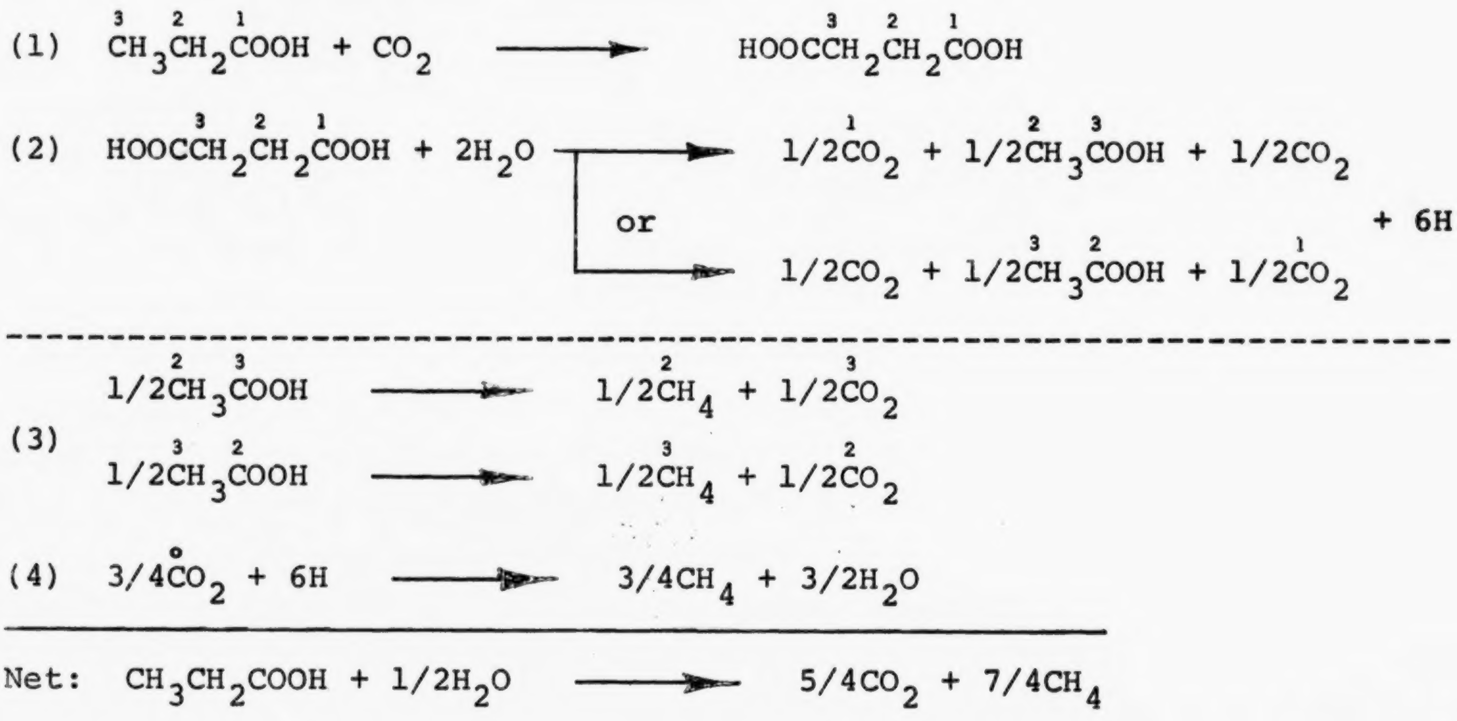
$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles } CO_2 \text{ reduced to } CH_4} = \frac{7/4}{3/4} = 2.33$$

If HPr-3C-14 is used:

$$SA\ \frac{CO_2}{CH_4} = 0$$

FIGURE 4

POSSIBLE PATHWAY FOR PROPIONIC ACID



(1) $\overset{3}{C}H_3\overset{2}{C}H_2\overset{1}{C}OOH + CO_2 \longrightarrow HOOC\overset{3}{C}H_2\overset{2}{C}H_2\overset{1}{C}OOH$

(2) $HOOC\overset{3}{C}H_2\overset{2}{C}H_2\overset{1}{C}OOH + 2H_2O \longrightarrow 1/2\overset{1}{C}O_2 + 1/2\overset{2}{C}H_3\overset{3}{C}OOH + 1/2CO_2$

or

$\longrightarrow 1/2CO_2 + 1/2\overset{3}{C}H_3\overset{2}{C}OOH + 1/2\overset{1}{C}O_2$ + 6H

---

(3) $1/2\overset{2}{C}H_3\overset{3}{C}OOH \longrightarrow 1/2\overset{2}{C}H_4 + 1/2\overset{3}{C}O_2$

$1/2\overset{3}{C}H_3\overset{2}{C}OOH \longrightarrow 1/2\overset{3}{C}H_4 + 1/2\overset{2}{C}O_2$

(4) $3/4\overset{\circ}{C}O_2 + 6H \longrightarrow 3/4CH_4 + 3/2H_2O$

Net: $CH_3CH_2COOH + 1/2H_2O \longrightarrow 5/4CO_2 + 7/4CH_4$

If $NaHC^{14}O_3$ or HPr-1C-14 was used:

$$\text{SA } \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles } CO_2 \text{ reduced to } CH_4} = \frac{7/4}{3/4} = 2.33$$

If HPr-2 or 3C-14 was used:

$$\text{SA } CO_2 = \frac{\text{Radioactive } CO_2}{\text{Total } CO_2} = \frac{5/16}{20/16} = .25$$

$$\text{SA } CH_4 = \frac{\text{Radioactive } CH_4}{\text{Total } CH_4} = \frac{11/16}{28/16} = .39$$

$$\text{SA } \frac{CO_2}{CH_4} = \frac{.25}{.39} = .64$$

∘ Indicates reducedradioactivity due to dilution with CO_2.

this analysis 5/16 and 11/16 moles of radioactive carbon dioxide and methane respectively would be produced. The specific activity of the methane is .39 and for carbon dioxide is .25. Thus, according to the mechanism presented in Figure 4 the specific activity ratio is .64 for carbons numbered 2 or 3. Use of labeled sodium bicarbonate or propionic acid-1C-14 would yield a specific activity ratio of 2.33 as in the previous analysis where only carbon dioxide reduction is involved in the formation of radioactive methane.

Further information may be obtained by feeding labeled butyrate to the propionic acid acclimated units. Butyrate was easily metabolized by the organisms in the propionate sludge as was shown in Warburg experiments which are reported later. As butyrate is an intermediate and has been shown to be degraded by the methane bacteria, any added information on its degradation could be of value. If the commonly accepted pathway of beta-oxidation was the primary mechanism, the use of butyric acid-2 or 4C-14 should yield only radioactive methane. However, with butyric acid-1 or 3C-14, both carbon dioxide and methane could become labeled, as radioactive carbon dioxide would first be evolved but a portion would be reduced to radioactive methane.

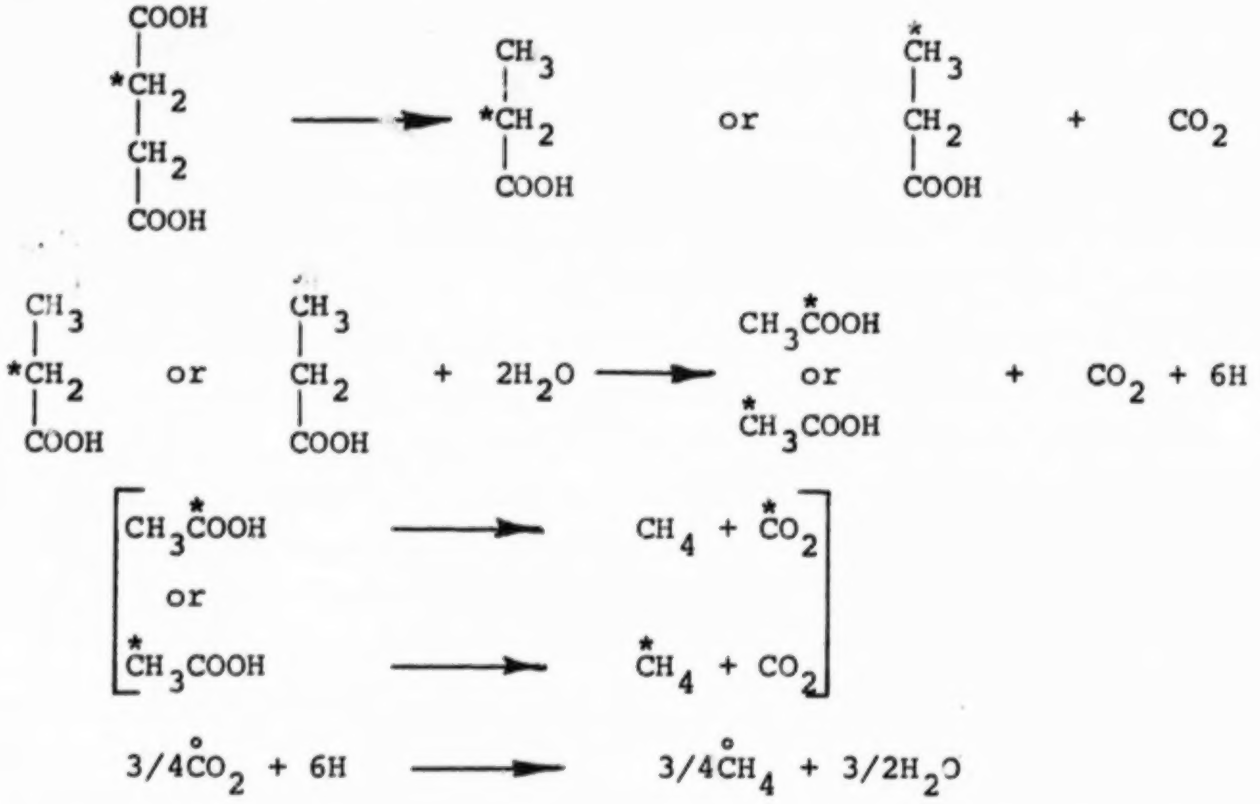
D. Succinic Acid

Succinic acid was studied since it is a symmetrical four carbon compound and an important intermediate in the tricarboxylic acid cycle. A possible pathway for the degradation of succinate is shown in Figure 5. In this process succinate is decarboxylated to propionate and is then converted to acetate which yields carbon dioxide and methane.

With the C-14 label in the #2 position of succinate, randomization occurs which allows the label to become either the #2 or #3 carbon in propionate. The symmetry of the succinate molecule allows decarboxylation to occur at either end. Accordingly one-half mole each of radioactive methane and carbon dioxide is produced from each mole of succinate and go into their respective pools. The activity of carbon dioxide becomes one-half radioactive mole per three moles total carbon dioxide produced or a specific activity of one-sixth (0.167). From each mole of succinic acid one-half mole each of radioactive and non-radioactive methane would be produced. An additional three-quarters moles of methane results from carbon dioxide reduction of which one-sixth or (3/4 X 1/6) are labeled. Thus fifteen-twentyfourths moles of radioactive methane are produced in a

FIGURE 5

POSSIBLE PATHWAY FOR SUCCINIC ACID



$$HOOC{-}\overset{*}{C}H_2{-}CH_2{-}COOH \longrightarrow CH_3{-}\overset{*}{C}H_2{-}COOH \text{ or } \overset{*}{C}H_3{-}CH_2{-}COOH + CO_2$$

$$CH_3{-}\overset{*}{C}H_2{-}COOH \text{ or } \overset{*}{C}H_3{-}CH_2{-}COOH + 2H_2O \longrightarrow CH_3\overset{*}{C}OOH \text{ or } \overset{*}{C}H_3COOH + CO_2 + 6H$$

$$\left[\begin{array}{lcl} CH_3\overset{*}{C}OOH & \longrightarrow & CH_4 + \overset{*}{C}O_2 \\ \text{or} & & \\ \overset{*}{C}H_3COOH & \longrightarrow & \overset{*}{C}H_4 + CO_2 \end{array}\right]$$

$$3/4\overset{\circ}{C}O_2 + 6H \longrightarrow 3/4\overset{\circ}{C}H_4 + 3/2H_2O$$

Net: $HOOCCH_2CH_2COOH + 1/2H_2O \longrightarrow 7/4CH_4 + 9/4CO_2$

If $NaHC^{14}O_3$ or succinic acid-1C-14 were used:

$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles } CO_2 \text{ reduced to } CH_4} = \frac{7/4}{3/4} = 2.33$$

If succinic acid-2 or 3C-14 were used:

$$SA\ CO_2 = \frac{\text{Radioactive } CO_2}{\text{Total } CO_2} = \frac{1}{6} = .167$$

$$SA\ CH_4 = \frac{\text{Radioactive } CH_4}{\text{Total } CH_4} = \frac{1/2 + 3/24}{7/4} = \frac{15/24}{7/4} = 0.357$$

$$SA\ \frac{CO_2}{CH_4} = \frac{.167}{.357} = 0.47$$

* indicates C^{14}

∘ Indicates reduced radioactivity due to dilution with CO_2.

total of seven-fourths moles of methane from each mole of succinic acid. The specific activity ratio of the carbon dioxide to methane would be .47.

If the pathway for succinic acid utilization passed through the citric acid cycle a major portion of the number two or three carbons of succinic acid would become methane. In such a case, using succinic acid-2 or 3C-14 the specific activity ratio of carbon dioxide to methane would approach zero.

When $NaHC^{14}O_3$ is used, the activity should be predominately in the carbon dioxide fraction. Any activity in the methane fraction comes from the reduction of carbon dioxide. The specific activity ratio should then be:

$$CO_2/CH_4 = \frac{\text{Total moles methane}}{\text{Moles } CO_2 \text{ reduced}} = 2.33$$

This result would also be applicable to the carboxyl carbons of succinate since decarboxylation occurs on both ends of the molecule. Thus, the label would be converted to carbon dioxide and the only label occurring in the methane could only result from carbon dioxide reduction.

E. Lactic Acid

Figures 1 and 6 illustrate a possible metabolic pathway for lactic acid. In the reactions shown in these figures, three moles of lactic acid are required to complete the series of reactions. One mole of lactic acid is dehydrogenated to yield pyruvic acid and then oxidized to acetate with the release of carbon dioxide and four hydrogen atoms (Eq. 1,2). Two moles of lactic acid are also dehydrogenated to yield two moles of pyruvic acid and then carboxylated to oxalacetate with the release of four more hydrogen atoms (Eq. 3,4). These eight hydrogen atoms reduce two moles of oxalacetate to yield succinate (Eq. 5).

Decarboxylation of succinate then occurs to yield propionate which is further oxidized to acetate and finally released as methane and carbon dioxide (Eq. 7,8).

From the reactions shown in Figure 1, carbon number 1 of lactic acid forms carbon dioxide and part of the carbon dioxide will be reduced to methane. While the fate of carbons #2 and #3 of lactate are much more complicated due to randomization when succinate is formed.

If $NaHC^{14}O_3$ or lactate-1C-14 is used or is formed during the degradation of lactic acid, a portion of the radioactive carbon dioxide will be reduced to radioactive methane. The specific activity ratio (SA) of carbon dioxide to methane based on the mechanism shown in Figure 6 may be

FIGURE 6

SUMMARY EQUATIONS OF LACTIC ACID FERMENTATION

$$CH_3CHOHCOOH \longrightarrow CH_3COCOOH + 2H \tag{1}$$
$$CH_3COCOOH + H_2O \longrightarrow CH_3COOH + CO_2 + 2H \tag{2}$$
$$2CH_3CHOHCOOH \longrightarrow 2CH_3COCOOH + 4H \tag{3}$$
$$2CH_3COCOOH + 2CO_2 \longrightarrow 2COOHCH_2COCOOH \tag{4}$$
$$2COOHCH_2COCOOH + 8H \longrightarrow 2COOHCH_2CH_2COOH + 2H_2O \tag{5}$$
$$2COOHCH_2CH_2COOH \longrightarrow 2CH_3CH_2COOH + 2CO_2 \tag{6}$$

NET: $$3CH_3CHOHCOOH \longrightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$$

$$2CH_3CH_2COOH + 4H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 12H \tag{7}$$
$$2CH_3COOH \longrightarrow 2CH_4 + 2CO_2 \tag{8}$$
$$3/2CO_2 + 12H \longrightarrow 3/2CH_4 + 3H_2O \tag{9}$$
$$CH_3COOH \longrightarrow CH_4 + CO_2 \tag{10}$$

NET: $$3CH_3CHOHCOOH \longrightarrow 9/2CO_2 + 9/2CH_4$$

If $NaHC^{14}O_3$ or lactic acid-1C-14 were used:

$$\text{Specific Activity (SA) } \frac{CO_2}{CH_4} = \frac{\text{Total } CH_4}{\text{Radioactive } CH_4} = 3.0$$

If lactic acid-2C-14 was used:

$$\text{SA } CO_2 = \frac{\text{Radioactive } CO_2}{\text{Total } CO_2} = \frac{2-1/2}{9/2} = .33$$

$$\text{SA } CH_4 = \frac{\text{Radioactive } CH_4}{\text{Total } CH_4} = \frac{1 + 1/2}{9/2} = .33$$

$$\text{SA } \frac{CO_2}{CH_4} = \frac{0.33}{0.33} = 1.0$$

If lactic acid-3C-14 was used:

$$\text{SA } CO_2 = \frac{\text{Radioactive } CO_2}{\text{Total } CO_2} = \frac{1-1/4}{9/2} = 0.167$$

$$\text{SA } CH_4 = \frac{\text{Radioactive } CH_4}{\text{Total } CH_4} = \frac{2 + 1/4}{9/2} = 0.50$$

$$\text{SA } \frac{CO_2}{CH_4} = \frac{0.167}{0.50} = 0.33$$

computed as follows:

$$SA \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles of } CO_2 \text{ reduced}} = \frac{9/2}{3/2} = 3$$

If lactate-2C-14 is used randomization occurs in two moles of lactate which allows the label to become either the #2 or #3 carbon in propionate. The symmetry of the succinate molecule allows decarboxylation to occur at either end. From three moles of lactate-2C-14 two moles of labeled carbon dioxide will result. However, one-half mole of the radioactive carbon dioxide will be reduced to labeled methane. One of the three moles of lactate would yield one mole of labeled methane. The computations of the specific activities of these gases is given in Figure 6.

With 3 moles of lactate-3C-14 two moles are randomized to allow the label to become either the #2 or #3 carbon in the propionate while the third mole of labeled lactate gives rise to labeled methane only. In this case two moles of labeled methane and one mole of labeled carbon dioxide are initially produced. For hydrogen balance, one fourth mole of radioactive carbon dioxide becomes labeled methane. The computations are given in Figure 6.

If the major degradative pathway were the direct reduction of lactate to propionate without the formation of succinate (35), lactate-3C-14 would yield only radioactive methane while lactate-1 or 2C-14 or $NaHC^{14}O_3$ would yield labeled carbon dioxide and methane (as a result of carbon dioxide reduction). The specific activity ratio of carbon dioxide to methane would be zero for lactate-3C-14 and 3.0 for the three other labeled compounds.

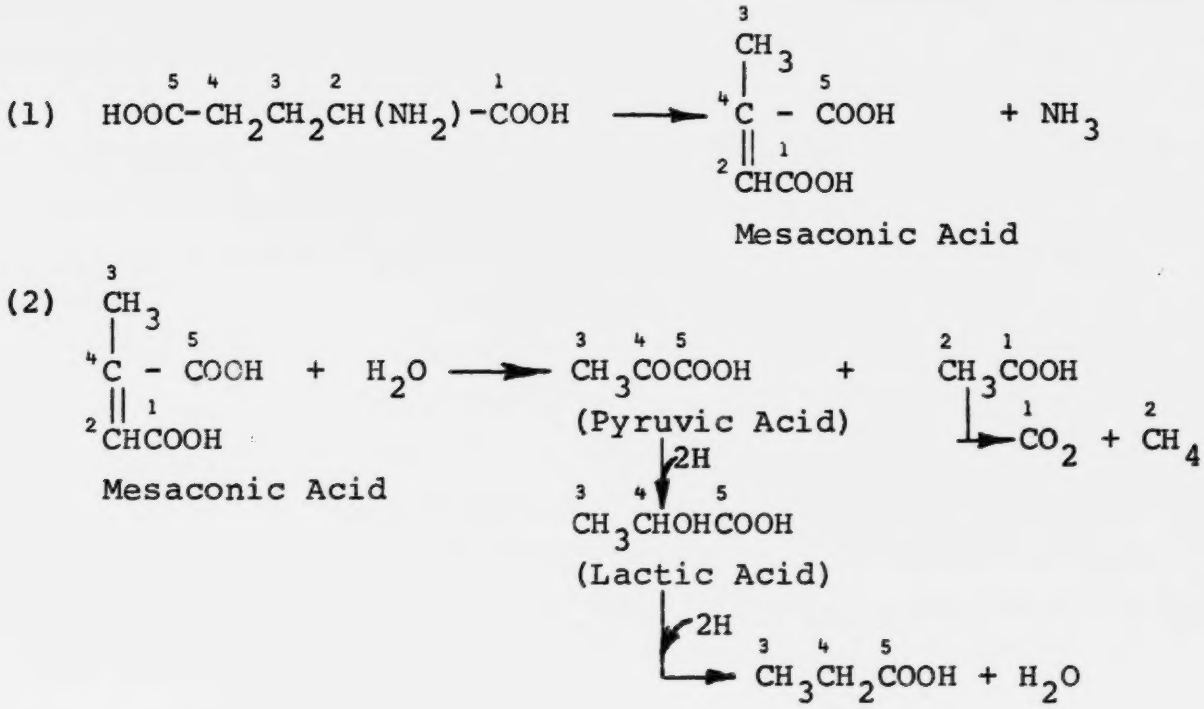
F. L(+) - Glutamic Acid

Presented in Figure 7 is a metabolic pathway for the degradation of L(+) - glutamic acid in anaerobic digestion.

Each mole of L(+) - glutamic acid is first degraded to mesaconic acid, and ammonia. Mesaconic acid is hydrolyzed to pyruvic acid and acetic acid. The acetic acid forms carbon dioxide and methane while pyruvic acid reacts with two hydrogen atoms and is thus reduced to lactic acid. The lactic acid also reacts with two hydrogen atoms and is reduced to propionic acid. The third equation shows the

FIGURE 7

POSSIBLE PATHWAY FOR GLUTAMIC ACID



(1) $$\overset{5}{\mathrm{HOOC}}-\overset{4}{\mathrm{CH_2}}\overset{3}{\mathrm{CH_2}}\overset{2}{\mathrm{CH}}(\mathrm{NH_2})-\overset{1}{\mathrm{COOH}} \longrightarrow \begin{array}{l} \overset{3}{\mathrm{CH_3}} \\ \;| \\ {}^{4}\mathrm{C} - \overset{5}{\mathrm{COOH}} \\ \;\|\;{}^{1} \\ {}^{2}\mathrm{CHCOOH} \end{array} + \mathrm{NH_3}$$

Mesaconic Acid

(2) $$\begin{array}{l} \overset{3}{\mathrm{CH_3}} \\ \;| \\ {}^{4}\mathrm{C} - \overset{5}{\mathrm{COOH}} \\ \;\|\;{}^{1} \\ {}^{2}\mathrm{CHCOOH} \end{array} + \mathrm{H_2O} \longrightarrow \overset{3}{\mathrm{CH_3}}\overset{4}{\mathrm{CO}}\overset{5}{\mathrm{COOH}} + \overset{2}{\mathrm{CH_3}}\overset{1}{\mathrm{COOH}}$$

Mesaconic Acid

(Pyruvic Acid)

$$\overset{2}{\mathrm{CH_3}}\overset{1}{\mathrm{COOH}} \longrightarrow \overset{1}{\mathrm{CO_2}} + \overset{2}{\mathrm{CH_4}}$$

$$\overset{3}{\mathrm{CH_3}}\overset{4}{\mathrm{CO}}\overset{5}{\mathrm{COOH}} \xrightarrow{2H} \overset{3}{\mathrm{CH_3}}\overset{4}{\mathrm{CHOH}}\overset{5}{\mathrm{COOH}}$$

(Lactic Acid)

$$\overset{3}{\mathrm{CH_3}}\overset{4}{\mathrm{CHOH}}\overset{5}{\mathrm{COOH}} \xrightarrow{2H} \overset{3}{\mathrm{CH_3}}\overset{4}{\mathrm{CH_2}}\overset{5}{\mathrm{COOH}} + \mathrm{H_2O}$$

(3) $\mathrm{CH_3CH_2COOH + 2H_2O \longrightarrow CH_3COOH + HCOOH + 4H}$

$\mathrm{CH_3COOH \longrightarrow CH_4 + CO_2}$

$\mathrm{HCOOH \longrightarrow 2H + CO_2}$

(4) $\mathrm{2H + 1/4CO_2 \longrightarrow 1/4CH_4 + 1/2H_2O}$

Net: $\mathrm{HOOC{-}CH_2CH_2CH(NH_2)COOH + 3/2H_2O \longrightarrow 11/4CO_2 + 9/4CH_4 + NH_3}$

conversion of propionic acid to acetic and formic acids and four hydrogen atoms. The acetic acid yields methane and carbon dioxide while the formic acid produces carbon dioxide and two hydrogen atoms. In the fourth equation, the reduction of carbon dioxide to methane is given.

From these reactions it can be seen that carbons numbered one, four, and five will form carbon dioxide and part of the carbon dioxide will be reduced to methane, while carbons numbered two and three will form methane only.

When C^{14} carbon dioxide is used or is formed during the degradation of L(+) - glutamic acid, a portion of the labeled carbon dioxide will be reduced to methane. The ratio of the specific activity of carbon dioxide to methane may be computed by the formula, previously derived, as follows:

$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles of } CO_2 \text{ reduced}} = \frac{9/4}{1/4} = 9$$

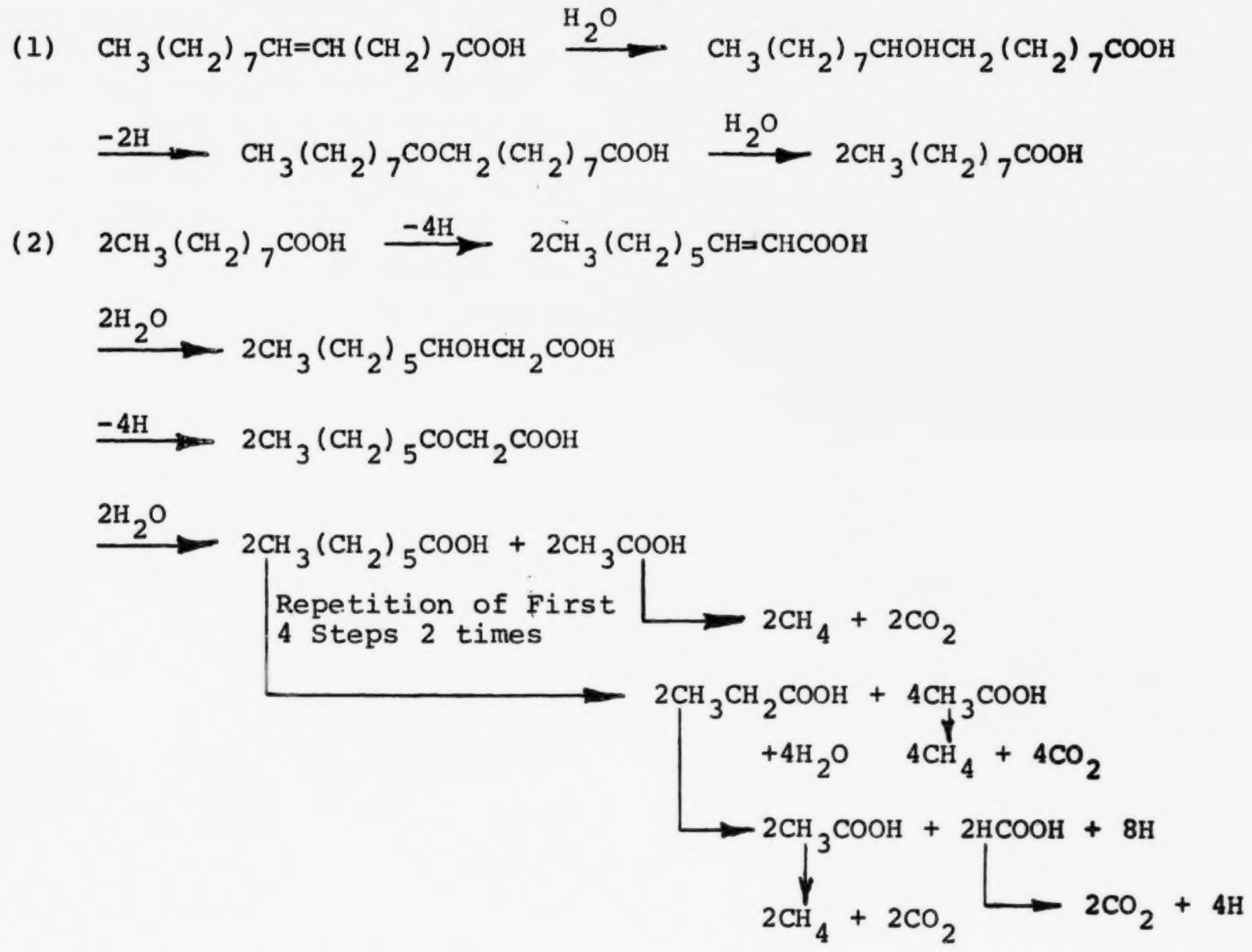
If the proposed pathway is correct, labeled sodium bicarbonate, DL-glutamic acid-1C-14, 4-C-14 or 5-C-14 will yield specific activity ratios of carbon dioxide to methane of 9. When DL - glutamic acid-2-C-14 or 3-C-14 are used, the ratios of the specific activity of carbon dioxide to methane will be zero since only methane becomes labeled.

G. Oleic Acid

Figures 8 and 9 have been prepared to illustrate two possible metabolic pathways for oleic acid in anaerobic digestion. In the reactions shown in Figure 8, each mole of oleic acid is hydrolyzed and oxidized to two moles of pelargonic acid prior to further degradation. The pelargonic acid is degraded by the beta oxidation process to acetic and propionic acids. Each mole of pelargonic acid forms three moles of acetic acid and one mole of propionic acid. The propionic acid is first converted to acetic acid, formic acid and four hydrogen atoms. The acetic acid forms methane and carbon dioxide while the formic acid yields carbon dioxide and two hydrogen atoms. Thirty eight hydrogen atoms are formed from each mole of oleic acid. The hydrogen atoms react with carbon dioxide producing methane.

FIGURE 8

POSSIBLE PATHWAY FOR OLEIC ACID



(1) $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH \xrightarrow{H_2O} CH_3(CH_2)_7CHOHCH_2(CH_2)_7COOH$

$\xrightarrow{-2H} CH_3(CH_2)_7COCH_2(CH_2)_7COOH \xrightarrow{H_2O} 2CH_3(CH_2)_7COOH$

(2) $2CH_3(CH_2)_7COOH \xrightarrow{-4H} 2CH_3(CH_2)_5CH{=}CHCOOH$

$\xrightarrow{2H_2O} 2CH_3(CH_2)_5CHOHCH_2COOH$

$\xrightarrow{-4H} 2CH_3(CH_2)_5COCH_2COOH$

$\xrightarrow{2H_2O} 2CH_3(CH_2)_5COOH + 2CH_3COOH$

$2CH_3COOH \rightarrow 2CH_4 + 2CO_2$

Repetition of First 4 Steps 2 times

$2CH_3(CH_2)_5COOH \rightarrow 2CH_3CH_2COOH + 4CH_3COOH$

$4CH_3COOH \rightarrow 4CH_4 + 4CO_2$

$2CH_3CH_2COOH + 4H_2O \rightarrow 2CH_3COOH + 2HCOOH + 8H$

$2CH_3COOH \rightarrow 2CH_4 + 2CO_2$

$2HCOOH \rightarrow 2CO_2 + 4H$

(1) $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH + 18H_2O \longrightarrow 8CH_4 + 10CO_2 + 38H$

(2) $38H + 19/4CO_2 \longrightarrow 19/4CH_4 + 19/2H_2O$

Net: $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH + 17/2H_2O \longrightarrow 51/4CH_4 + 21/4CO_2$

If labeled sodium bicarbonate or oleic acid-1C-14 are used:

$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles radioactive } CH_4} = \frac{51/4}{19/4} = 2.68$$

When C^{14} carbon dioxide is used or is formed during the degradation of oleic acid, a portion of the radioactive carbon dioxide will be reduced to radioactive methane. The specific activity ratio of carbon dioxide to methane based on the mechanism shown in Figure 8 may be computed by the formula previously derived as follows:

$$\text{S.A. } \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles } CO_2 \text{ reduced to } CH_4} = \frac{51/4}{19/4} = 2.68$$

In the pathway proposed in Figure 9 each mole of oleic acid is first reduced by two hydrogen atoms to stearic acid prior to further degradation. The stearic acid is degraded through beta-oxidation to acetic acid before finally being degraded to carbon dioxide and methane. In Figure 9 an odd and an even carbon are shown as labeled. Following the reaction with the number two carbon labeled, it is found that only methane becomes labeled and the carbon dioxide is completely unlabeled. This would be true for all the even numbered carbons of stearic acid or any other even-carbon saturated fatty acid fermented by beta-oxidation. The ratio of the specific activity of carbon dioxide to methane for all even numbered carbons would thus be zero.

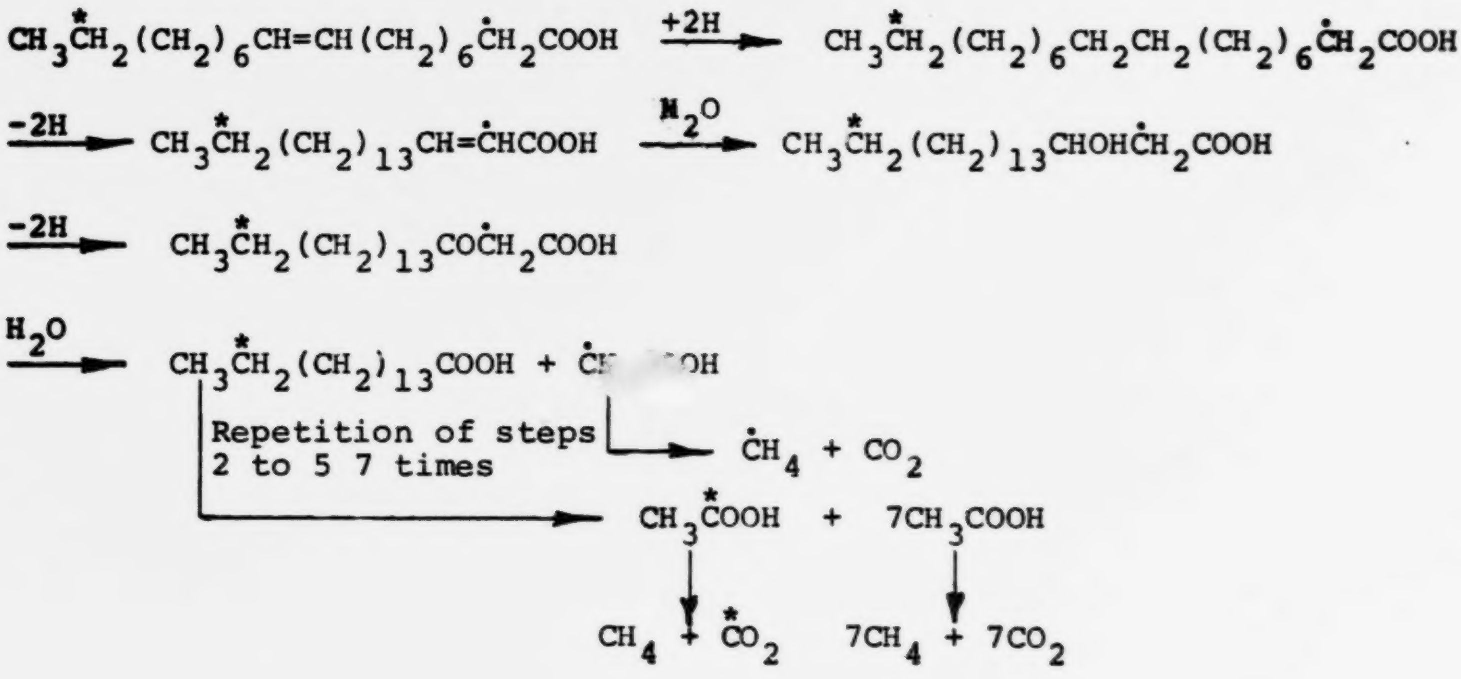
With the number seventeen carbon labeled or with any odd numbered carbon, only the carbon dioxide formed would be labeled. However, some of the carbon dioxide would be reduced to methane. The specific activity ratio of carbon dioxide to methane can be computed by the same formula previously derived and is shown in Figure 9.

If labeled sodium bicarbonate were used instead of labeled oleic acid, labeled methane could only be produced by carbon dioxide reduction. The same would be true for the beta-oxidation of oleic acid-17-C^{14}.

Jeris and McCarty (17) concluded that in the degradation of medium and long-chain saturated fatty acids omega-oxidation plays a secondary role, if any at all; similarly it is not believed that oleic acid is degraded through the omega-oxidation process.

FIGURE 9

POSSIBLE PATHWAY FOR OLEIC ACID



$CH_3\overset{*}{C}H_2(CH_2)_6CH{=}CH(CH_2)_6\dot{C}H_2COOH \xrightarrow{+2H} CH_3\overset{*}{C}H_2(CH_2)_6CH_2CH_2(CH_2)_6\dot{C}H_2COOH$

$\xrightarrow{-2H} CH_3\overset{*}{C}H_2(CH_2)_{13}CH{=}\dot{C}HCOOH \xrightarrow{H_2O} CH_3\overset{*}{C}H_2(CH_2)_{13}CHOH\dot{C}H_2COOH$

$\xrightarrow{-2H} CH_3\overset{*}{C}H_2(CH_2)_{13}CO\dot{C}H_2COOH$

$\xrightarrow{H_2O} CH_3\overset{*}{C}H_2(CH_2)_{13}COOH + \dot{C}$[illegible]OH

Repetition of steps
2 to 5 7 times

$\dot{C}H_4 + CO_2$

$CH_3\overset{*}{C}OOH + 7CH_3COOH$

$CH_4 + \overset{*}{C}O_2 \quad 7CH_4 + 7CO_2$

(1) $CH_3\overset{*}{C}H_2(CH_2)_6CH{=}CH(CH_2)_7COOH + 16H_2O \longrightarrow 9CH_4 + 8CO_2 + \overset{*}{C}O_2 + 30H$

(2) $30H + 15/4\overset{\circ}{C}O_2 \longrightarrow 15/4\overset{\circ}{C}H_4 + 15/2H_2O$

Net: $CH_3\overset{*}{C}H_2(CH_2)_6CH{=}CH(CH_2)_7COOH + 17/2H_2O \longrightarrow 15/4\overset{\circ}{C}H_4 + 9CH_4 + 21/4CO_2$

$$SA\ \frac{CO_2}{CH_4} = \frac{\text{Total moles of } CH_4}{\text{Moles radioactive } CH_4} = \frac{15/4 + 9}{15/4} = 3.40$$

* and · indicate C^{14}

° Indicates reduced radioactivity due to dilution with CO_2.

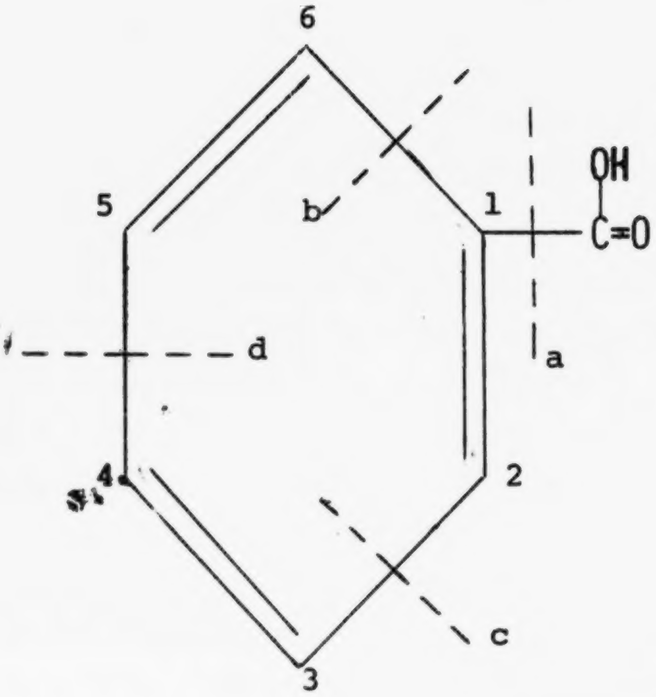
H. Benzoic Acid

As indicated in the literature survey most of the mechanisms for cleavage of the benzene ring require oxygen. Fina and Fiskin (26) however, have shown that mixed cultures of methane bacteria are quantitatively able to ferment benzoate to methane and carbon dioxide. In this case an electron acceptor other than oxygen would have to be supplied but it is likely that the points of ring cleavage would be the same as in aerobic fermentation. Figure 10 illustrates the most likely sites of ring cleavage and their sequence. The final products would be succinate and acetate. The conversion ratio in the final cleavage would be one mole of succinate to 2 moles of acetate. Although this cannot be stated with absolute certainty further degradation of succinate would be necessary for utilization by the methane formers.

The net result shown in Figure 10 is the quantitative conversion of benzoate to acetate. Each mole of benzoate yields 3 moles of acetate which in turn are converted to methane and carbon dioxide. With the carbon label in the #1 position only methane becomes labeled while the #7 position would yield only labeled carbon dioxide, except for the carbon dioxide reduced to methane as required for hydrogen balance.

FIGURE 10

POSSIBLE PATHWAY FOR BENZOIC ACID



Sequence

a - Decarboxylation

b,c,d - Ring cleavage points

$$C_7H_6O_2 + 6H_2O \longrightarrow 3CH_3COOH + CO_2 + 6H$$

$$\overset{1}{C}H_3\overset{2}{C}OOH \longrightarrow \overset{1}{C}H_4 + \overset{2}{C}O_2$$

$$2CH_3COOH \longrightarrow 2CH_4 + 2CO_2$$

$$3/4\overset{0}{C}O_2 + 6H \longrightarrow 3/4\overset{0}{C}H_4 + 3/2H_2O$$

$$\text{Net: } C_7H_6O_2 + 9/2H_2O \longrightarrow 13/4CO_2 + 15/4CH_4$$

If $NaHC^{14}O_3$ or Benzoate-7C-14 is used:

$$SA\frac{CO_2}{CH_4} = \frac{\text{total moles } CH_4}{\text{moles radioactive } CH_4} = \frac{15/4}{3/4} = 5$$

If Benzoate-1C-14 is used:

$$SA\frac{CO_2}{CH_4} = 0$$

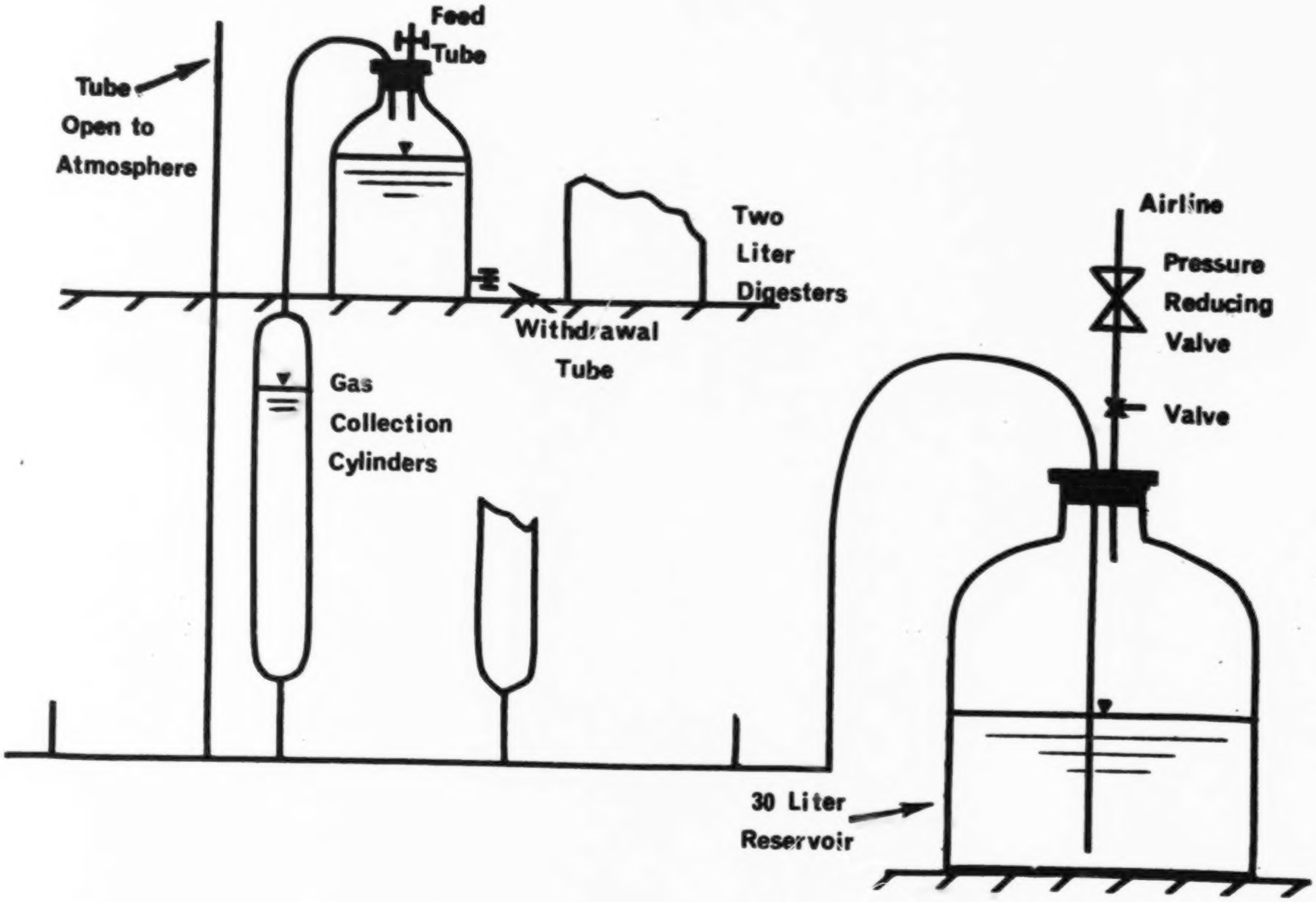
IV. EXPERIMENTAL PROCEDURES AND APPARATUS

A. Description of Apparatus

1. Digesters

The object of this study was to determine the biochemical mechanisms by which certain organic compounds are degraded in the anaerobic methane fermentation using C^{14} tracer materials and other chemical methods. Laboratory digesters were slug fed daily in a manner resembling the typical operation of field installations. These laboratory digesters consisted of two-liter aspirator glass bottles as shown in Figure 11. A short rubber tubing was placed on the bottom port with a clamp. Through this opening sludge was withdrawn and samples were taken for analysis. A two-holed rubber stopper was inserted to seal the contents of the bottle from the atmosphere. Through one hole was inserted a short piece of glass tube with rubber tubing on the exposed end, sealed with a tight pinch clamp. This tubing was used for feeding and the expulsion of gases. Through the other hole in the rubber stopper another short glass tube was inserted. The exterior end of this tubing was connected to a 1300 ml glass gas collection cylinder by means of tygon tubing.

The 1300 ml glass gas collection cylinder was calibrated for measuring gas production. This cylinder was connected by 3/8" tygon tubing to a header. The 1/2" tygon header was connected to all the gas collection cylinders and led to a 30-liter reservoir which contained the gas confining fluid. To insure a low solubility for carbon dioxide the confining fluid was composed of a saturated solution of sodium chloride in 10 percent sulfuric acid. In order to facilitate the readings of the gas volume, the confining fluid was colored using methyl orange. The 30-liter reservoir consisted of a narrow-mouthed pyrex bottle in which was inserted a two-holed rubber stopper. One hole was connected to the tygon header tubing which extended to the bottom of the reservoir. Through the other hole a short piece of glass tube was inserted and connected to a rubber tube leading to a three-way valve followed by a pressure reducing valve which was connected to a low pressure air system. When the pressure was introduced on the confining fluid system all the digesters were under pressure and a sample of sludge or gas could be withdrawn. When



BATCH FED DIGESTER SYSTEM

FIGURE 11

the pressure was released, a vacuum, created on the digester system facilitated feeding.

All digesters were kept in a walk-in room in which the temperature was held constant at 35° ± 1°C. The heat was supplied by a portable electric heater and regulated by a mercury thermoregulator. Furthermore, a small fan was operated continuously to provide an even distribution of heat.

Before conducting a one-day slug feed radioactive study, the contents of the digester was kept continuously mixed for 2 to 4 days by using a gas recirculating pump. The pressure side of the pump was connected to the port at the bottom of the digester while the vacuum side was fitted to the gas collection cylinder. The gas pump was a Dyna-pump, Neptune Model No. 3 having a capacity of 430 cubic inches per minute at a maximum pressure of 14 psi. For long term studies in which the isotope was fed daily for more than 30 days, a magnetic mixer was used rather than a gas pump to mix the digester contents.

2. Gas Analysis and Collection Apparatus

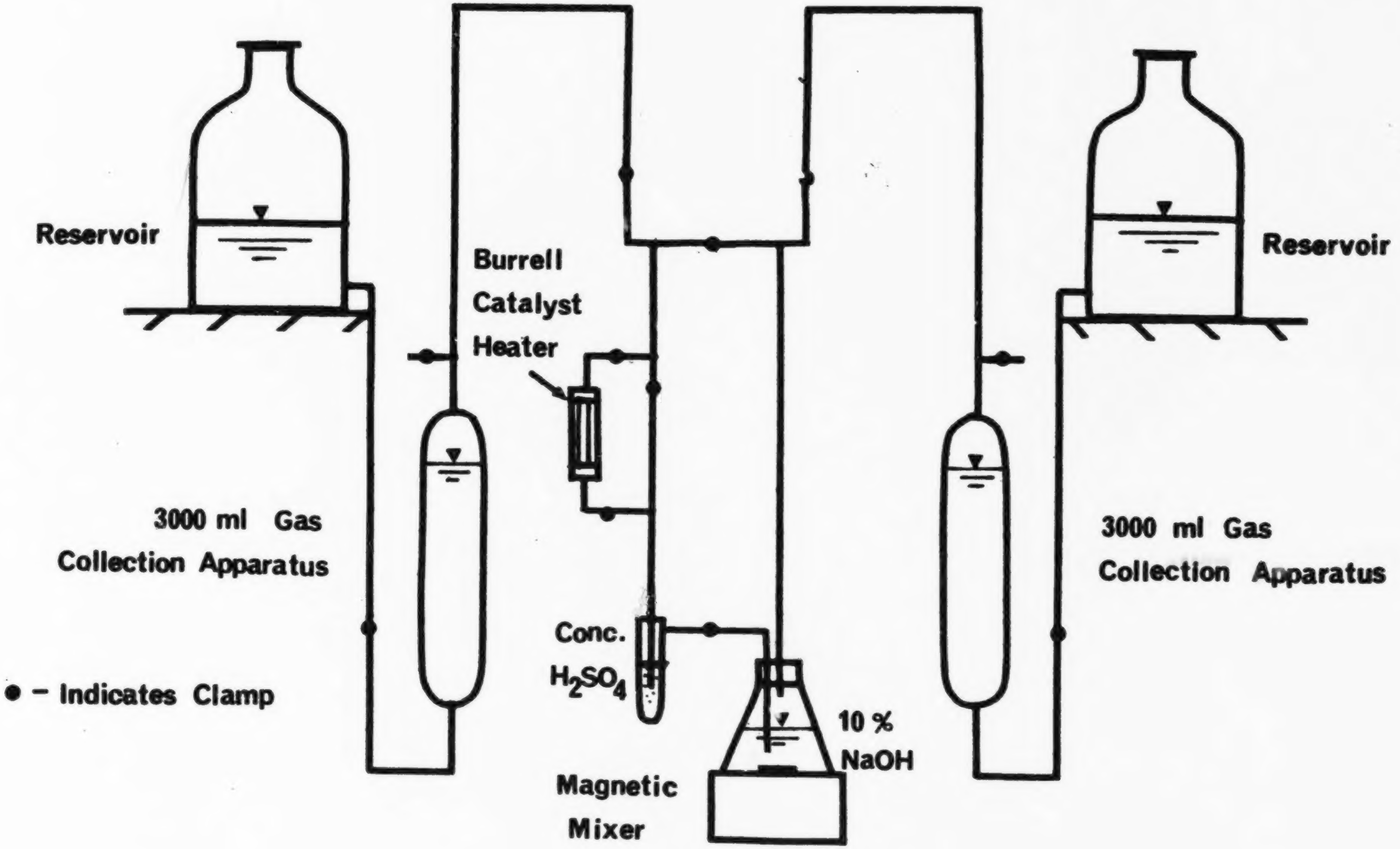
Carbon dioxide, methane and nitrogen were measured with an Aerograph Gas Chromatograph, Model A-90-P, using Silica gel and molecular sieve columns, and was connected to a Honeywell recorder.

Carbon dioxide and methane was collected from the digesters with 100, 1000, or 3000 ml. glass cylinders. One end of the glass cylinder was connected with a short piece of rubber tubing and sealed with a pinch clamp. The other end of the glass cylinder was fitted with a rubber tubing and connected to an aspirator bottle that contained confining fluid.

The separation of carbon dioxide and methane was accomplished by bubbling the gas through a water solution containing 10 percent sodium hydroxide. A trap containing concentrated sulfuric acid was used to absorb the water vapor present in the gas. The methane was oxidized to carbon dioxide using a Burrell catalyst heater. The carbon dioxide thus obtained was also collected in a 10 percent sodium hydroxide solution. The carbon dioxide and methane separating apparatus is shown in Figure 12.

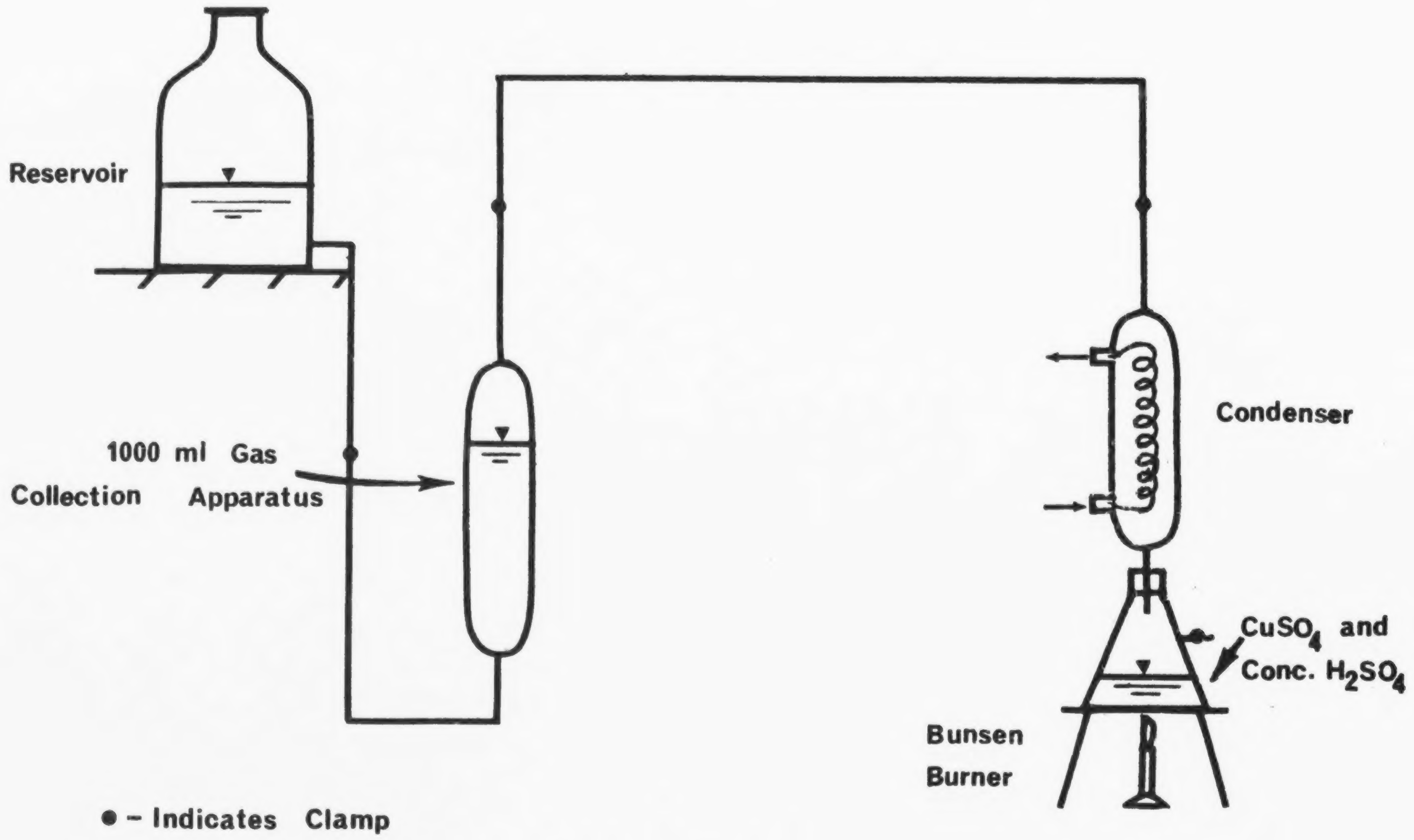
3. Sludge Boiling Apparatus for Collecting Soluble Carbon Dioxide

The apparatus shown in Figure 13 was used to boil sludge and collect the gas evolved. The apparatus was evacuated by use of a



CARBON DIOXIDE AND METHANE SEPARATION APPARATUS

FIGURE 12



SLUDGE BOILING APPARATUS FOR EXTRACTING CARBON DIOXIDE

FIGURE 13

vacuum pump. Sludge was first taken in a separatory funnel which was previously filled with nitrogen and allowed to flow into the erlenmeyer through the side port. Sludge boiling was accomplished in the erlenmeyer flask which was connected to a condenser followed by a 1000 ml gas collection cylinder. One-half gram of cupric sulfate and 20 ml of concentrated sulfuric acid were placed in the erlenmeyer flask before sludge was added. Cupric sulfate was added in order to fix any sulfide present, while sulfuric acid was used to destroy all the alkalinity. Sludge boiling was accomplished by a Bunsen burner.

4. Carbon Dioxide Preparation Apparatus

The apparatus shown in Figure 14 was used for the preparation of carbon dioxide for counting radioactivity. Acidification of the sodium hydroxide-sodium carbonate solution was accomplished by means of the buret filled with concentrated sulfuric acid and controlled by a glass stopcock. Upon acid addition, carbon dioxide was evolved and it was collected in the 1000 ml gas collection cylinder. The carbon dioxide was then absorbed in the phenethylamine (PEA) solution in a 50 ml erlenmeyer flask. Mixing of the PEA solution was facilitated by a magnetic stirrer. A 100 ml gas collection cylinder was also connected to the 50 ml erlenmeyer. Before carbon dioxide was absorbed in the PEA solution, the air in the erlenmeyer was transferred into this 100 ml gas collection cylinder. The composition of the PEA solution (CO_2 absorbent mixture and scintillation chemicals) was as follows:

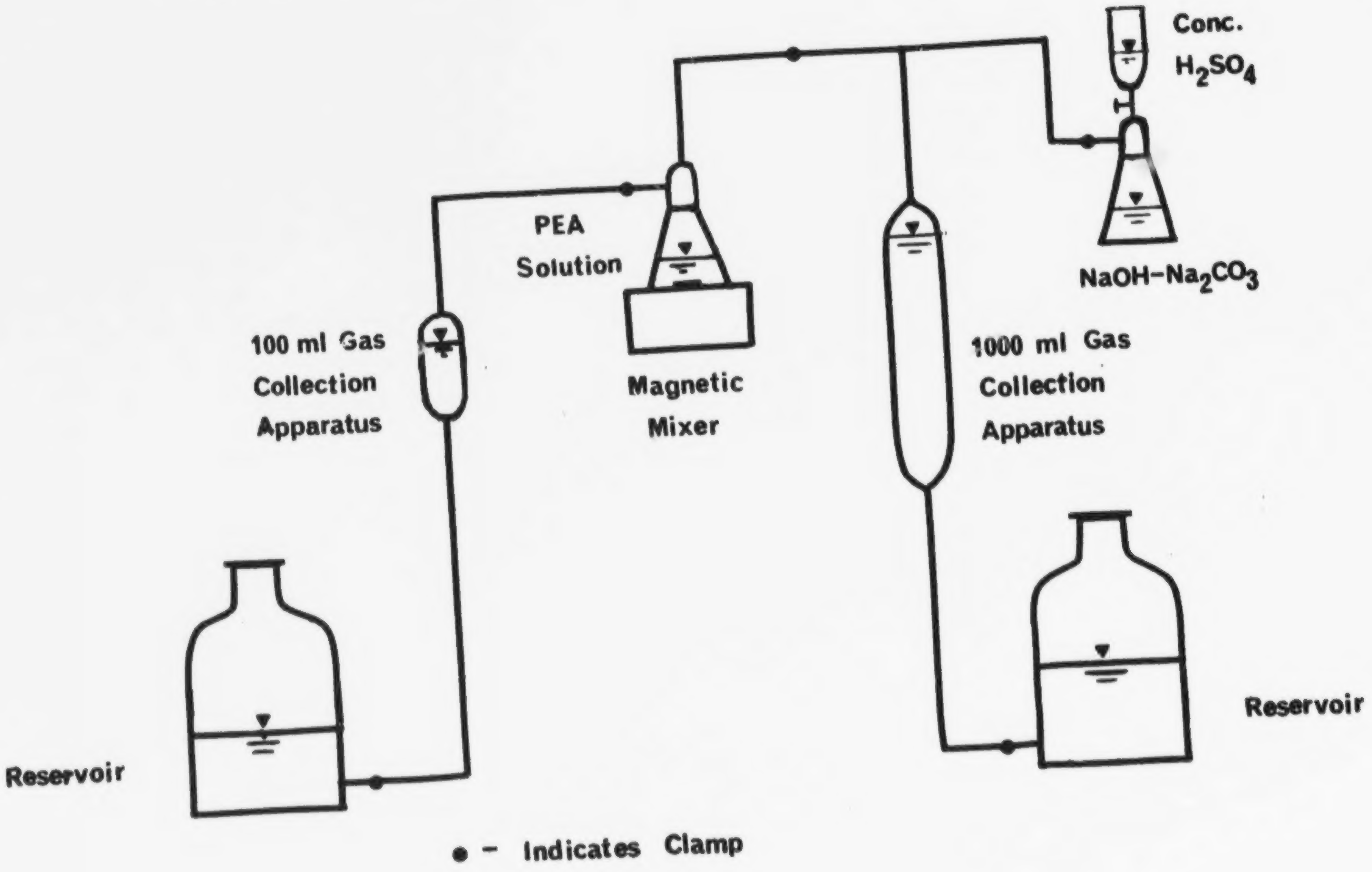
CO_2 Absorbent-Scintillation Mixture

50.0 mg [POPOP] Dimethyl 1,4 bis-2-(4-methyl-5-phenyl oxazolylbenzene)
2500 mg [PPO] 2,5 diphenyl oxazole
Add 150-200 ml Toluene and mix
135.0 ml Methanol (Absolute)
135.0 ml Phenethylamine
Dilute to 500.0 ml with Toluene

After the PEA solution was saturated or a measured amount of carbon dioxide had been absorbed, the PEA solution was poured into

FIGURE 14

APPARATUS FOR CARBON DIOXIDE ABSORPTION



a plastic vial and placed into the scintillation counter.

5. Radio Assay Equipment

An automatic tri-carb liquid scintillation spectrometer, model 314-C as manufactured by Packard Instrument Company, Inc. was used for the counting of carbon dioxide.

B. Experimental Procedure

The experimental procedures used were designed to yield well operating anaerobic digesters for the study of certain biochemical mechanisms. All digester units were slug fed once daily simulating the conditions present at typical field installations. When equilibrium was reached, selected radioactive compounds were added and the gases were collected and counted as previously described. An excess of nitrogen, phosphorus and other nutrients was maintained in the anaerobic units at all times. In order to check the effectiveness of the digester operation, routine chemical analyses were performed during the studies. These analyses included pH, alkalinity, volatile acids, COD and gas analysis.

Volatile acid intermediates and end products were determined by gas-liquid chromatography and chemical methods. The utilization of substrate was computed on the basis of the COD fed and COD of the mixed effluent and also from the methane production.

1. Selection of Substrate

The compounds selected for study were either known components of sewage sludge, possible intermediates or metabolites active in carbohydrate, fat or protein breakdown.

Selection of the C^{14} label in these compounds was based on the availability and known reactions of these compounds in biological systems.

Successful biological growth requires certain inorganic nutrients for the synthesis of new cell mass. To satisfy these requirements nitrogen, phosphorus and trace quantities of several other elements such as potassium, magnesium, iron and calcium were added to the daily feed. These inorganic nutrients were maintained as two stock solutions. At all times an excess of all inorganic nutrients was maintained in the anaerobic units in order that optimum growth could be attained. The composition of these stock solutions is given in Table 1.

TABLE 1

NUTRIENT SOLUTIONS

Nutrient 4A	Nutrient M
45.6 gm $(NH_4)_2HPO_4$	28 gm NH_4Cl
diluted to 1 liter with distilled water	10 gm $MgCl_2 \cdot 6H_2O$
	5 gm $FeCl_3$
	4 gm KCl
	1.12 gm $AlCl_3 \cdot 6H_2O$
	1.0 gm $CaCl_2 \cdot 2H_2O$
	0.57 gm $CoCl_2 \cdot 6H_2O$
	0.25 gm $MnSO_4 \cdot H_2O$
	0.1 gm $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$
	0.01 gm $ZnCl_2$
	diluted to 1 liter with distilled water.

2. Start-up and Purging of Digesters

All units were originally initiated from fresh sludge obtained from local plants or from older units already in operation in the laboratory. At least two months of operation was allowed before studies were made in order to insure biological equilibrium and the purging of the original feed from the digester. All digesters were maintained at 1500 ml sludge volume and a 15 day detention time.

3. Operation of Digesters

Every day between nine o'clock a.m. and twelve o'clock noon a gas reading was taken, a 100 ml digester sample was withdrawn and 100 ml of substrate were added. The digesters were well shaken prior to withdrawal of sample to insure uniformity of the sample, the shaken again after feeding of the substrate. During the day, the digesters were shaken once or twice to allow good contact to take place between the microorganisms and the substrate.

The amount of COD fed varied with the ability of the biological system to degrade the substrate.

Since some compounds, notably the dicarboxylic acids, are not easily dissolved in distilled water but do dissolve easily as the sodium salt, they were used in this form. Excess sodium hydroxide was neutralized using hydrochloric acid in order to maintain a neutral pH and a reasonable alkalinity.

When the feed concentration of oleic acid exceeds 500 mg/l COD, it necessitates the addition of calcium chloride to diminish the toxicity of oleic acid to the biological system. For every mole of oleic acid over 500 mg COD, 1/2 mole of calcium chloride was added.

Feeding was accomplished by using volumetric pipettes. A separate pipette was used to feed each substrate. Pipettes used for feeding radioactive substances were carefully washed after each feeding and were kept separate from the other pipettes.

Occassionally the digestion was unsatisfactory and the feed concentration of substrate was decreased until proper operation was restored. Infrequently supernatant liquor powder from the Yonkers, New York anaerobic digesters was added to poorly operating digesters in order to stimulate the process.

The gas distribution in the gas and liquid phase had to be at equilibrium before proper data could be obtained. Equilibrium in pH, alkalinity, volatile acids, and COD of the mixed effluent was also desirable during the test period. Constancy of gas production and mixed effluent COD indicated the equilibrium in substrate utilization.

C. Analytical Techniques

Generally the techniques as outlined in the 12th edition of Standard Methods (27) were followed. Any procedural modifications are described below.

1. pH Determination

A Corning Model 12 pH meter with glass and calomel electrodes were used for this determination. The procedure followed is as described in Standard Methods (27). Prompt analysis and careful handling of samples reduced the loss of carbon dioxide and resultant pH change.

2. Alkalinity

The procedure for waste water described in Standard Methods (27) was followed. Additional procedures and modifications are as follows; a) the concentration of the sulfuric acid used in titration was 1.0N; b) a magnetic stirrer was used during titration to provide mixing of the liquor; c) a 50 ml sample was used; d) the end point of titration was pH 4.0 and the titration was determined with a Corning Model 12 pH meter.

3. Volatile Acids

The procedure described in the 11th edition of Standard Methods (28) was followed. The following modifications were made: a) fifty ml samples were used; b) the samples were diluted to 200 ml with sulfuric acid solution which was prepared by dilution of 190 ml concentrated H_2SO_4 to 11.0 liters with tap water.

McCarty (29) found that for acetic acid, 65 percent is recovered in the distillate. Therefore, the recovery factor used in the calculations of volatile acids concentration was 0.65.

During the later phases of the work, the Column Partition Chromatography Method (Silicic Acid Modification) was used. The procedure followed is described in the 12th edition of Standard Methods (27) with the modification that 100 ml of chloroform - butanol reagent were used.

4. Chemical Oxygen Demand

The rapid method reported by Jeris (30) for determining COD was followed. Due to the large concentration of chlorides in some of the sludge samples, 0.8 grams of mercuric sulfate was used in these analyses.

5. Gas Collection and Analysis

The procedure for gas collection and analysis is outlined as follows:

a. Preparation of the carbon dioxide and methane separating apparatus (Figure 12).

  1) About 7 ml of concentrated sulfuric acid was added to the test tube trap.
  2) Twenty-five ml of saturated sodium hydroxide solution and 75 ml of distilled water were added to a 125 ml flask. A magnetic bar was also placed in the flask.
  3) Prepurified nitrogen gas was used to purge all the CO_2 from the glass tubing of the apparatus.
  4) All the openings were clamped to prevent CO_2 from the atmosphere from entering the system.

b. Preparation of the sludge boiling apparatus. (Figure 13).

  1) The gas collection cylinder was filled with the confining fluid to the clamp at the top of the cylinder.
  2) Cold water was allowed to flow through the condenser.

3) One half gram of cupric sulfate and 20 ml of concentrated sulfuric acid were placed in the erlenmeyer flask. The erlenmeyer flask was connected to the condenser.

4) The vacuum pump was turned on, evacuating all the air in the erlenmeyer flask.

c. Preparation for gas collection and analysis.

1) A 3000 ml gas collection cylinder was filled with confining fluid.

2) The separatory funnel was filled with nitrogen gas.

3) One and one-half liters of warm water were prepared (about 35°C).

d. Gas collection and analysis.

1) Eighty ml gas was collected from the digester in a 100 ml gas collection cylinder.

2) Two 50 ml liquid samples were taken from the digester.

3) Four hundred ml of digester liquor were placed directly from the digester into the separatory funnel without exposing them to the atmosphere.

4) The gas was collected from the digester with the 3000 ml gas collection cylinder. After all the gases from the digester system were collected, the tygon tubing was clamped at the top of the 1300 ml gas collection cylinder to prevent the confining fluid from flowing into the digester.

5) The warm water was added to the digester through the port at the bottom of the digester to displace all of the gas into the 3000 ml gas collection cylinder.

6) The gas collected was analyzed using an Aerograph Gas Chromatograph, Model A-90-P. The gas used for flushing the sampling port of the gas chromatograph was recovered.

7) The sludge liquor sample was analyzed for pH, alkalinity, volatile acids and COD.

e. Gas collection from digester liquor and analysis.

1) Allow 350 ml of liquor to flow into the evacuated erlenmeyer flask (Part b). A bunsen burner was used to heat the liquor.

2) The gas evolved was collected in a cylinder.

    3) After all the gases had evolved from the boiling liquor, boiling water was placed into the erlenmeyer flask to displace all gases into the calibrated gas collection cylinder.
    4) The gaseous volume was recorded and a gas analysis using the chromatograph was made. The gas used for flushing the gas chromatograph sampling valve was also recovered.
    5) The gases collected from the boiled sludge were placed into the 3000 ml gas collection cylinder for later CO_2 absorption in sodium hydroxide solution.

f. The gases collected in the 3000 ml gas collection cylinder were bubbled through sodium hydroxide solution several times to remove all of the carbon dioxide (Figure 12). A magnetic stirrer was used to facilitate the absorption of the carbon dioxide.

g. After all the carbon dioxide had been absorbed in sodium hydroxide solution, pure oxygen was added to the 3000 ml gas collection cylinder for combusting the methane and thus converting it into carbon dioxide. Generally, two volumes of oxygen are required for each volume of methane present for this conversion. In this analysis excess of oxygen was added to insure complete combustion.

h. The combustion was accomplished using a Burrell catalyst heater.

i. The mixture of methane and oxygen was passed through the Burrell catalyst heater. Water was removed in the sulfuric acid trap and the carbon dioxide was collected in a new sodium hydroxide solution. A magnetic stirrer was also used.

j. Preparation of carbon dioxide for counting (Figure 14).
    1) Carbon dioxide was bubbled into one-half of the confining fluid contained in the aspirator bottle in the carbon dioxide preparation apparatus so that half the confining fluid was saturated with carbon dioxide.
    2) The sodium hydroxide-sodium carbonate solution (from f above) was transferred into a flask which was tightly fitted by a burette.
    3) Sufficient concentrated sulfuric acid was added to the burette for acidification and slowly added to the flask.

4) About 900 ml of carbon dioxide were collected in the 1000 ml gas collection cylinder from the above acidification.

5) The exact volume of carbon dioxide collected in the cylinder was recorded.

6) Twenty ml of PEA solution were added to a 50 ml erlenmeyer flask. A magnetic bar was also placed into this erlenmeyer flask.

7) The air in the erlenmeyer flask was transferred into the 100 ml gas collection cylinder.

8) The magnetic stirrer was activated to facilitate rapid absorption of carbon dioxide in PEA solution.

9) The exact volume of carbon dioxide absorbed in PEA solution was measured.

k. The PEA solution was placed into a plastic vial and then placed into the Packard Tri-Carb Scintillation counter for counting the radioactivity of carbon dioxide.

l. Slides of the digester liquor were prepared.

m. Forty ml of sludge and 40 ml of volatile acid distillate were stored for later identification of volatile acid intermediates.

6. Identification of Volatile Acids

In order to determine individual volatile acids present in the digesters, a Varian Aerograph 600-C chromatograph equipped with a hydrogen flame ionization detector was used. This detector is insensitive to water, thus permitting use of aqueous samples. The hydrogen gas source was provided by an Aerograph model 650 Hydrogen generator. The conditions and column used for the instrument were the following:

Carrier Gas - Nitrogen - 25ml/min
Column Temperature - 165°C
Injector Temperature - 195°C
Hydrogen Flow rate - 20ml/min
Column - NPGS, 2% H_3PO_4, 60/80 Firebrick, 5' X 1/4" S.S.
Sample size - 0.7 µl

In addition, a Brown-Honeywell recorder with a 1 mv range and ten inch chart was used to record the signal.

The instrument was standardized using known concentrations of acetic, propionic, normal and isobutyric, normal and isovaleric acids. Samples were injected in 0.7 microliter quantities using a Hamilton microliter syringe. Excellent results were obtained in analyzing digester contents using this procedure.

7. Anaerobic Warburg Operation

The procedure followed has been previously reported (31, 32). Additional procedures and modifications included the following: a) one hundred and twenty-five ml flasks without center wells, but fitted with side inlets and side wells were used; b) a 2 or 3 ml volume of feed solution was added to the side wells; c) the 2 or 3 ml feed solution stored in the side wells was poured into the flasks after the manifold connecting the manometers had been removed; d) the 2 or 3 ml of feed solution plus 50 ml of sludge gave a mixed volume of 52 or 53 ml.

V. RESULTS

A. Radioactive Tracer Studies

The biochemistry of the methane fermentation was studied using various radioactively labeled carbon compounds. In Table 2 are listed the substrates fed and the radioactive isotopes used with these substrates.

TABLE 2

SUBSTRATES AND RADIOISOTOPES USED

Propionic Acid

HPr-1C-14
HPr-2C-14
HPr-3C-14
HBu-2C-14
HBu-3C-14
$NaHC^{14}O_3$

Succinic Acid

HSu-2C-14
$NaHC^{14}O_3$

Lactic Acid

HLa-1C-14
HLa-2C-14
HLa-3C-14
$NaHC^{14}O_3$

Glutamic Acid

HGl-1C-14
HGl-2C-14
HGl-5C-14
$NaHC^{14}O_3$

Oleic Acid

HOl-1C-14
$NaHC^{14}O_3$

Benzoic Acid

HBe-1C-14
HBe-7C-14
$NaHC^{14}O_3$

In these studies radioactivity was slug fed one time to some of the digester units while it was slug fed to all the other units daily for at least a 30-day period prior to collection of gases for radioactivity analyses. All the substrates were slug fed on a daily basis and the digestion detention period was 15 days for all units. At least two runs were conducted on each substrate and labeled compound used.

All units were operated over an extended period of time prior to collecting gas samples for radio assay. This period served to acclimate the biological population to the substrate studied and allow equilibrium conditions to be established. During each run gas production and composition were determined. The gas volumes were reported at atmospheric pressure and 35°C. Other tests performed which served to establish the operating characteristics for each run included; pH, alkalinity, volatile acids, and COD. The efficiency of substrate utilization was determined by computing the COD of the feed and determining the average mixed COD of the effluent. As a check on the reliability of the data, a balance of the COD input and output was made. The only COD input was the feed. The output included the mixed effluent and methane gas produced. These computations were based on the gas volume and analyses performed which are outlined in the "Sample Calculations" section of the Appendix.

1. Propionic Acid

Table 3 gives a summary of the results of the tracer investigation on propionic acid. Included in this table is the COD reduction based on two methods of calculation which are outlined in the Appendix. Also, the experimental specific activity ratio of carbon dioxide to methane is given along with the theoretical value predicted by the metabolic pathways given in Figures 3 and 4. As the results vary considerably in the tracer studies, each is discussed separately.

a) Propionic Acid-2C-14

Two digester runs were performed in this tracer series. Radioactivity was slug fed daily in the two runs for more than 30 days. The COD reduction of the fed substrate averaged approximately 96 percent. The COD balances, when comparing COD reduction based on chemical analysis and on the quantity of methane produced, were in good agreement and within a range of 5 to 9 percent. The experimental ratios of the activity of carbon dioxide to methane were 1.25 and 1.34. The two values were between those predicted by the pathways shown in Figures 3 and 4 indicating both mechanisms may occur in the degradative process.

b) Propionic Acid-3C-14

Five digester runs were made in this tracer series. Activity was slug fed once only in three of the five runs and slug fed daily in the other two runs for more than 30 days. The COD analyses for two of the five runs were not made. The COD balances were from 2 to 10 percent apart for the other runs and gave COD

TABLE 3

SUMMARY OF PROPIONIC ACID

	% COD Reduction		Activity CO_2/CH_4			% CH_4 from Acetic Acid	
C-14 Label	Chem Basis	CH_4 Basis	Exp.	Fig.3	Fig.4	Exp.	Theo.
Propionic Acid-2C-14	96.0	87.1	1.25	2.33	.64	-	-
	96.5	91.3	1.34	2.33	.64	-	-
Propionic Acid-3C-14	-	-	0.03*	0	.64	-	-
	-	-	0.28*	0	.64	-	-
	95.7	86.0	0.41	0	.64	-	-
	96.5	94.3	0.60*	0	.64	-	-
	96.8	87.4	0.83	0	.64	-	-
$NaHC^{14}O_3$	93.6	91.7	1.91	2.33	2.33	47.6	57.0
	-	50.6	2.18*	2.33	2.33	54.2	57.0
	92.6	88.7	2.07*	2.33	2.33	64.5	57.0
Butyric Acid-2C-14	93.3	89.0	0.53	-	-	-	-
	97.0	79.9	1.08	-	-	-	-
	94.8	95.5	1.22	-	-	-	-
	96.2	79.2	1.39	-	-	-	-
Butyric Acid-3C-14	87.5	75.4	1.68	-	-	-	-
Propionic Acid-3C-14	Fresh	Sludge	0.96^1	-	-	-	-
	Fresh	Sludge	0.99^1	-	-	-	-

* Isotope sludge fed 1 day only.

[1] Ratio of total radioactivity in CO_2 to total activity in CH_4.

reductions of 96, 97, and 97 percent respectively. The experimental ratios of the activity of carbon dioxide to methane were; 0.03, 0.28, 0.41, 0.60 and 0.83. The first two values compared well to the predicted results for the pathway postulated in Figure 3. The other results were spread from 0.41 to 0.83 indicating strongly the probability of randomization.

c) Propionic Acid-$NaHC^{14}O_3$

Three digester runs were made in this tracer study series. Activity was slug fed one time in two of the three runs and slug fed daily in the other runs for 36 days. The COD reductions for these three runs were approximately 93, 51, and 91 percent respectively. The COD balance was excellent in two of these runs as there was only a 2 to 4 percent difference. The COD reduction based on a chemical basis for the other run was not made.

The experimental results gave specific activity ratios of carbon dioxide to methane of 1.91, 2.18, and 2.07, compared with the predicted value of 2.33.

d) Butyric Acid-2 or 3C-14 Fed to Propionate Acclimated Digesters

Results of the studies using butyric acid-2C-14 varied from 0.53 to 1.39 indicating various pathways may have been involved in the degradation. For 3C-14 butyrate only one study was run, which yielded a specific activity ratio of 1.68; however, as the activity of the gases counted were only two times greater than background, this result is not considered satisfactory. In this tracer study series, the COD reduction based on chemical analyses ranged from 87 to 97 percent.

e) Propionic Acid-3C-14 Fed to Fresh Digested Sludge

Digesting sludge taken from a well operating anaerobic digester at the Yonkers, New York primary treatment plant was placed in a two liter bottle in the same manner and detail as previously reported. After two days of satisfactory gas production based on a raw sludge feed, propionic acid-3C-14 was added. Gas collection and analyses were also made in the manner previously described. In the two runs conducted, the ratio of the total radioactivity in the carbon dioxide to that in the methane were 0.96 and 0.99.

2. Succinic Acid

A tabulation of the radiostudy results for succinic acid is presented in Table 4. In all of these studies the labeled compound was fed to the digester for more than 30 days prior to the collection of gases for radioactivity analyses. Extended control analyses were performed during the last 3-4 days prior to the collection of gases.

TABLE 4

SUMMARY OF SUCCINIC ACID STUDY

C^{14} Label	COD Reduction Chem. Basis	COD Reduction CH_4 Basis	Activity CO_2/CH_4 Exp.	Activity CO_2/CH_4 Theo.	% CH_4 from Acetic Acid Exp.	% CH_4 from Acetic Acid Theo.
Succinic Acid-2C-14	92.8	99.5	1.00	0.47	-	-
"	92.2	90.6	1.13	0.47	-	-
"	92.6	83.8	1.17	0.47	-	-
"	95.8	84.4	1.36	0.47	-	-
$NaHC^{14}O_3$	92.4	104	2.48	2.33	59.7	57.0
"	95.3	109	2.50	2.33	60.0	57.0
"	94.1	99.8	2.13	2.33	53.0	57.0
"	95.3	99.7	2.03	2.33	49.5	57.0

a) Succinic Acid-2C-14

Results given in Table 4 for succinic acid-2C-14 indicate that the number two carbon of succinic acid yields a considerable amount of radioactive carbon dioxide and does not appear to follow the pathway given in Figure 5. The four results presented range between 1.00 and 1.36 which is a relatively narrow range. Substrate utilization based on COD reduction extended from 84 to 99 percent and is considered satisfactory.

b) Succinic Acid-$NaHC^{14}O_3$

Results from the experiments with C-14 labeled bicarbonate indicate that the extent of carbon dioxide reduction to methane is about one mole for every 2.33 moles of methane produced. The actual ratios were 2.48, 2.50, 2.13 and 2.03. These results are in reasonable agreement with the predicted quantity.

3. Lactic Acid

In this radioactive tracer study four different C-14 labeled compounds were used. Prior to each test run, extensive data on the performance of the anaerobic digesters were collected for at least five days under equilibrium conditions. Table 5 gives a summary of the results obtained in these runs.

TABLE 5

SUMMARY OF LACTIC ACID STUDY

	% COD Reduction		Activity CO_2/CH_4		% CH_4 from Acetic Acid	
C^{14} Label	Chem. Basis	CH_4 Basis	Exp.	Theo. (Fig. 6)	Exp.	Theo.
$NaHC^{14}O_3$	94.7	88.7	2.57	3	61.1	66.7
$NaHC^{14}O_3$	94.5	83.0	2.85	3	64.9	66.7
Lactic Acid-1C-14	93.4	79.3	2.77	3	-	-
Lactic Acid-1C-14	93.6	79.6	2.92	3	-	-
Lactic Acid-2C-14	94.0	97.2	1.14	1.0	-	-
Lactic Acid-3C-14	96.4	99.5	1.46	0.33	-	-
Lactic Acid-3C-14	94.3	92.5	1.18	0.33	-	-
Lactic Acid-3C-14	95.0	96.2	1.0	0.33	-	-
Lactic Acid-3C-14	95.0	90.0	1.06	0.33	-	-
Lactic Acid-3C-14	87.5	85.9	0.32	0.33	-	-

a) Lactic Acid-1C-14

Using this labeled compound, two runs were made with two different digesters. In both cases the radioactivity was slug fed one time. The COD reduction of the substrate fed based on chemical analysis was slightly greater than 93 percent. However, only 79 percent COD reduction was obtained based on methane production. The specific activity ratios of carbon dioxide to methane obtained were 2.77 and 2.92, comparing very favorably to the theoretical value of 3.0 proposed in Figure 6.

b) Lactic Acid-2C-14

One run was made using the number 2 carbon of lactic acid radioactively labeled. The C-14 compound was slug fed for 46 consecutive days before the gases produced were collected for analysis. Substrate COD reduction efficiency was 94 percent based on chemical analysis and 97 percent based on methane production. The specific activity ratio of carbon dioxide to methane was 1.14 and compared well to the theoretical value given in Figure 6.

c) Lactic Acid-3C-14

In this series of five digester runs the COD efficiencies of substrate utilization ranged from 86 to 99 percent based on both chemical analysis and methane production. Radioactivity in the form of lactic acid-3C-14 was slug fed daily for at least 60 days prior to collection and analysis of the gases produced. The experimental ratios of the radioactivity of carbon dioxide to methane were; 1.46, 1.18, 1.0, 1,06 and 0.32 as compared to the theoretical value of 0.33 given in Figure 6.

d) Lactic Acid-$NaHC^{14}O_3$

Two digester runs were made feeding $NaHC^{14}O_3$ to the lactic acid acclimated digesters. Radioactivity was slug fed one time in both of the runs. The COD reductions based on chemical analyses were slightly over 95 percent and ranged from 83 to 89 percent based on methane production. The experimental specific activity ratios of carbon dioxide to methane were 2.57 and 2.85 and compared reasonably well to the theoretically expected ratio of 3.0. Since nearly two-thirds of the methane produced came from acetic acid degradation, one mole of the acetic acid must have been produced from each mole of lactic acid utilized.

4. Glutamic Acid

Ten separate runs were made with four different tracer compounds in order to determine the mode of degradation of this important amino acid. Radioactive bicarbonate tests were conducted four times using three digester units. Duplicate units were operated on each of the other three radioactive tracers used. Activity was slug fed one time to three of the four units fed $NaHC^{14}O_3$ while it was slug fed to all the other seven units for at least a 30-day period prior to collection of gases for radioactivity analyses. Since the results given in Table 6 vary considerably for each tracer study, each is discussed separately.

TABLE 6

SUMMARY OF GLUTAMIC ACID STUDY

C^{14} Label	%COD Reduction		Activity CO_2/CH_4		$\%CH_4$ From Acetic Acid	
	Chem. Basis	CH_4 Basis	Exp.	Theo.	Exp.	Theo.
DL-Glutamic Acid-1C-14	94.3	98.0	9.20	9.00	-	-
	90.7	85.4	8.69	9.00	-	-
DL-Glutamic Acid-2C-14	97.1	91.0	0.19	0.00	-	-
	95.0	95.4	0.12	0.00	-	-
DL-Glutamic Acid-5C-14	93.8	81.2	9.02	9.00	-	-
	95.4	81.0	9.81	9.00	-	-
$NaHC^{14}O_3$	95.7	89.7	7.01*	9.00	85.8	88.9
	96.0	98.5	8.94*	9.00	88.8	88.9
	88.1	81.0	8.09*	9.00	87.6	88.9
	71.2	68.0	9.00	9.00	88.9	88.9

* Indicates isotope was fed for 1 day only.

a) DL-Glutamic Acid-1 and 5C-14

The COD balance was in fair agreement in the 1C-14 studies but was from 13 to 14 percent apart in the 5C-14 runs. Approximately 90 percent COD reduction was obtained in this series of four runs. The experimental results of the ratio of the activity of carbon dioxide to methane for the DL-glutamic acid-1 and 5C-14 studies were confined to a relatively narrow range of 8.69 to 9.81. These values compared very well to the theoretical results.

b) DL-Glutamic Acid 2C-14

The COD reduction for both units averaged close to 95 percent and the COD balance was good. The ratios of the activity of carbon dioxide to methane were 0.19 and 0.12 in the two tests, comparing well with the predicted value of zero. The low specific activity ratio indicates that most of the activity was in the methane fraction.

c) L(+)-Glutamic Acid-$NaHC^{14}O_3$

Four digester runs were made in this tracer series. Radioactivity was slug fed one time in three of the four runs and slug fed daily in the other run for 117 days. The COD reductions based on chemical analysis for these four runs were approximately 96, 96, 88, and 71 percent respectively. The COD balance was good in these runs and showed a difference of 3 to 7 percent when compared to the COD reduction based on methane production. The experimental ratios of the activity of carbon dioxide to methane were 7.01, 8.94, 8.09, and 9.00, comparing very favorably with the theoretical results predicted. As only one (11-14%) out of every nine moles of methane produced came from carbon dioxide reduction, the proposed pathway given in Figure 7 is in agreement with the results.

5. Oleic Acid-1C-14 and $NaHC^{14}O_3$

Four individual tests were made with two different tracer compounds. Duplicate units were run on each of the radioactive tracers used. The tracer compounds were fed daily to all units for at least 30 days prior to collection to gases for radioactivity analyses. The final test data were collected over periods extending from the last four to six days prior to collection of gases. Results of these runs are shown in summary Table 7.

TABLE 7

SUMMARY OF OLEIC ACID STUDY

	%COD Reduction		Activity CO_2/CH_4			%CH_4 From Acetic Acid	
C^{14} Label	Chem. Basis	CH_4 Basis	Exp.	Fig 8	Fig 9	Exp.	Theo.
Oleic Acid-1C-14	84.6	88.1	3.00	2.68	3.40	-	-
	89.9	84.5	3.49	2.68	3.40	-	-
$NaHC^{14}O_3$	97.9	104.3	4.08	2.68	3.40	75.5	70.6
	88.6	81.8	3.48	2.68	3.40	71.3	70.6

The COD reduction for all units averaged close to 90 percent. The COD balances were in fair agreement and within a range of 4 to 7 percent. The experimental results of the ratio of the activity of carbon dioxide to methane for the labeled bicarbonate and oleic acid-1C-14 were 4.08, 3.48, 3.49, and 3.00. The first three values compared favorably to the predicted results for the pathway postulated in Figure 8, but differed markedly from the pathway shown in Figure 9. The last value was approximately the arithmetic mean of the two theoretical results which were 2.68 and 3.40, indicating that both pathways may have been involved as degradative mechanisms in this run.

6. Benzoic Acid

A number of digesters were fed benzoic acid to test the ability of an anaerobically grown mixed culture to break an aromatic ring. Table 8 presents a summary of the results of the research on benzoic acid. For simplicity and ease in interpretation, all the radio tracers used will be discussed together and will include benzoic acid-1C-14, 7C-14 and $NaHC^{14}O_3$.

TABLE 8

SUMMARY OF BENZOIC ACID STUDY

C^{14} Label	%COD Reduction Chem. Basis	%COD Reduction CH_4 Basis	Activity CO_2/CH_4 Exp.	Activity CO_2/CH_4 Theo.	%CH_4 From Acetic Acid Exp.	%CH_4 From Acetic Acid Theo.
Benzoic Acid-1C-14	83	102.4	0.057	0	-	-
	91.6	83.5	0.18	0	-	-
Benzoic Acid-7C-14	89.6	97.0	3.88	<5	-	-
	94.3	89.0	3.53	<5	-	-
$NaHC^{14}O_3$	94.3	110	5.5	5	81.8	80.0
	93.7	83.8	4.6	5	78.2	80.0
	-	-	4.71	5	78.8	80.0
	100.0	101.0	5.64	5	82.3	80.0
	91.3	95.0	5.0	5	80.0	80.0

Two digester runs each were made using the two benzoic acid C-14 labeled compounds and five runs using $NaHC^{14}O_3$. All three tracers were slug fed one time in these runs. COD reduction ranged from 83 to 100 percent, based on chemical analysis and from 83 to 110 percent based on methane production equivalent.

The specific activity ratio of carbon dioxide to methane for the benzoic acid-1C-14 studies were 0.057 and 0.18 indicating most of the activity is in the methane fraction. Thus, the number 1 carbon yields essentially 100 percent methane as postulated in Figure 10. The experimental results of the ratios of the activity of carbon dioxide to methane for the benzoic acid-7C-14 were 3.88 and 3.53, while for the $NaHC^{14}O_3$ they ranged between 4.6 to 5.6. The results indicate a major concentration of activity in the carbon dioxide and also show a minor difference between exogenous carbon dioxide ($NaHCO_3$) and the endogenous carboxyl carbon of benzoic acid.

B. Identification of Volatile Acid Intermediates

During the course of the investigation it became desirable to determine the individual volatile acids present in each digester. It was believed that identification of the various products formed would be helpful in interpreting some of the C-14 tracer studies. Samples were collected from the various digesters throughout the length of the investigation. In this way analyses included samples from all substrates under varying digester conditions. Numerous samples were analyzed with a hydrogen flame ionization chromatograph as previously described.

The instrument was standardized using standard solutions of acetic, propionic, butyric, isobutryic, valeric and isovaleric acids. A listing of the acids found in each digester analyzed in this phase of the study had concentrations of volatile acids extending from 0 to 2,000 mg/l depending on the condition of the digesters and is presented in Table 9.

TABLE 9

VOLATILE ACID INTERMEDIATES

Acid Substrate	ACIDS PRESENT IN DIGESTERS			
	Acetic	Propionic	Butyric	Valeric
Propionic	M	M	m	None
Succinic	m	M	None	None
Lactic	M	M	m	m
Glutamic	M	m	None	None
Oleic	M	m	None	None
Benzoic	M	m	None	None

m = minor acid <100 mg/l
M = major acid >100 mg/l

1. Propionic Acid

One-hundred twenty-five mixed effluent samples from propionic acid acclimated digesters were analyzed for individual volatile acids. The concentration of the acids present, expressed as acetic acid, extended from 0 to 2000 mg/l. Acetic and propionic acids were always present in the greatest concentration. Butyric acid was present in forty-five of the samples but in small concentrations. Isobutyric and valeric acid were found in two samples.

2. Succinic Acid

Volatile acid intermediate studies performed on the succinate units indicate that the major acid is propionic with a smaller amount of acetic acid present. The concentration of acetic acid remained below 100 mg/l, indicating that propionic acid conversion to acetic acid is the rate-limiting step in the formation of methane and carbon dioxide.

3. Lactic Acid

Propionic and acetic acid were present in considerable concentrations during the time that lactic acid was used as the substrate. Normal valeric, butyric and isovaleric acids were also present in comparably smaller amounts and were considered to be of minor significance. Valeric acid was assumed to be synthesized by condensation of acetic and propionic acid molecules, while formation of butyric acid was probably due to condensation of two acetic acid molecules.

4. Glutamic Acid

In the 36 glutamic acid digester samples tested, the range in volatile acid extended from 12 to 1500 mg/l as acetic acid. Of these samples, 32 had less than 300 mg/l and one sample greater than 1000 mg/l. Acetic acid was found to be present in the greatest concentration in all samples analyzed. Propionic acid was also found present in 35 of the 36 samples analyzed but always below 100 mg/l concentration, and less than 50 mg/l in all but three samples. No higher acid was found in any of the samples analyzed.

5. Oleic Acid

The range of volatile acids concentration in the oleic acid acclimated digesters extended from zero to 340 mg/l as acetic acid. It was found that acetic acid was always present in the greatest concentration. In 5 of the 24 samples analyzed propionic acid was found in trace amounts. No higher acids were found in any of the samples tested.

6. Benzoic Acid

Of the 22 samples analyzed for volatile acids in the benzoic acid acclimated digesters, seven had acetic acid in concentrations extending from 17 to 360 mg/l and three had propionic acid with 25 to 156 mg/l.

C. Anaerobic Warburg Experiments

Anaerobic Warburg experiments were conducted on various sludge liquors according to the experimental procedures previously described. The aim of these experiments was to determine which compounds were possible intermediates in the degradation of the substrates fed to the digesters. It was hoped that compounds which were readily utilized by the sludge liquor would provide evidence for the mechanism involved in the degradation of the substrates to which the digesters had been acclimated.

The gas produced in the first few hours of these tests was due principally to an acid-alkalinity reaction rather than biological action. Chemical analyses including pH, alkalinity, volatile acids, total and soluble COD were conducted before and after the completion of each test. The gas production during the Warburg runs was corrected by subtracting the gas production from the unfed controls and adjusting for the change in the solubility of carbon dioxide due to the varied percentage of carbon dioxide in the gas phase in the flask. The efficiency of substrate utilization was determined by COD analyses. These data and results are given in the Appendix along with the typical calculations used to obtain the results.

1. Propionic Acid-Runs 1 and 2

Two Warburg experiments were made using sludge liquor acclimated to propionic acid. The gas production curves for these runs are given in Figures 15 and 16.

a) Warburg Run 1

In Run 1 acetic, propionic, lactic and succinic acids were studied. The pH of the flasks was maintained between 7.1 and 7.3

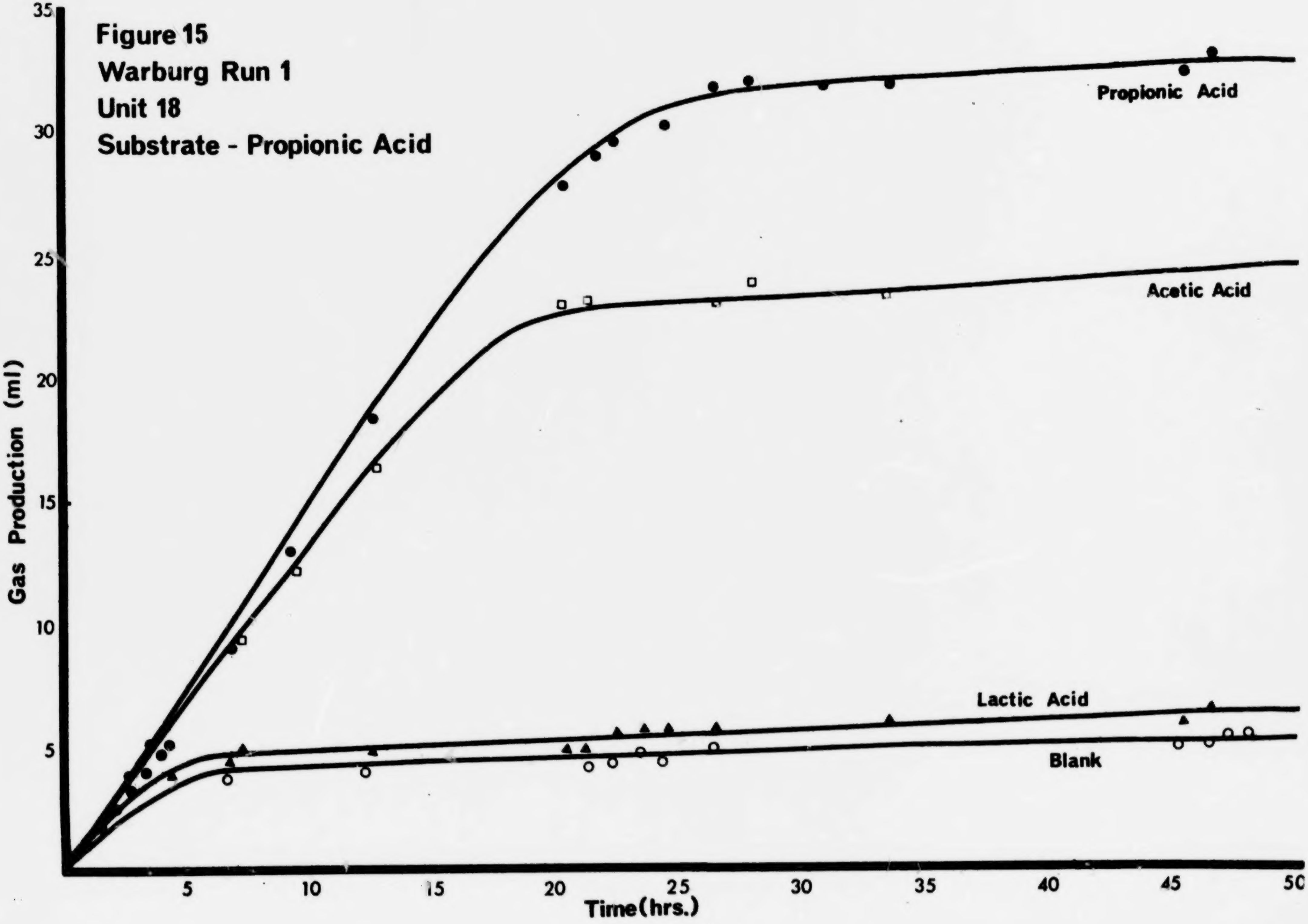


Figure 15
Warburg Run 1
Unit 18
Substrate - Propionic Acid

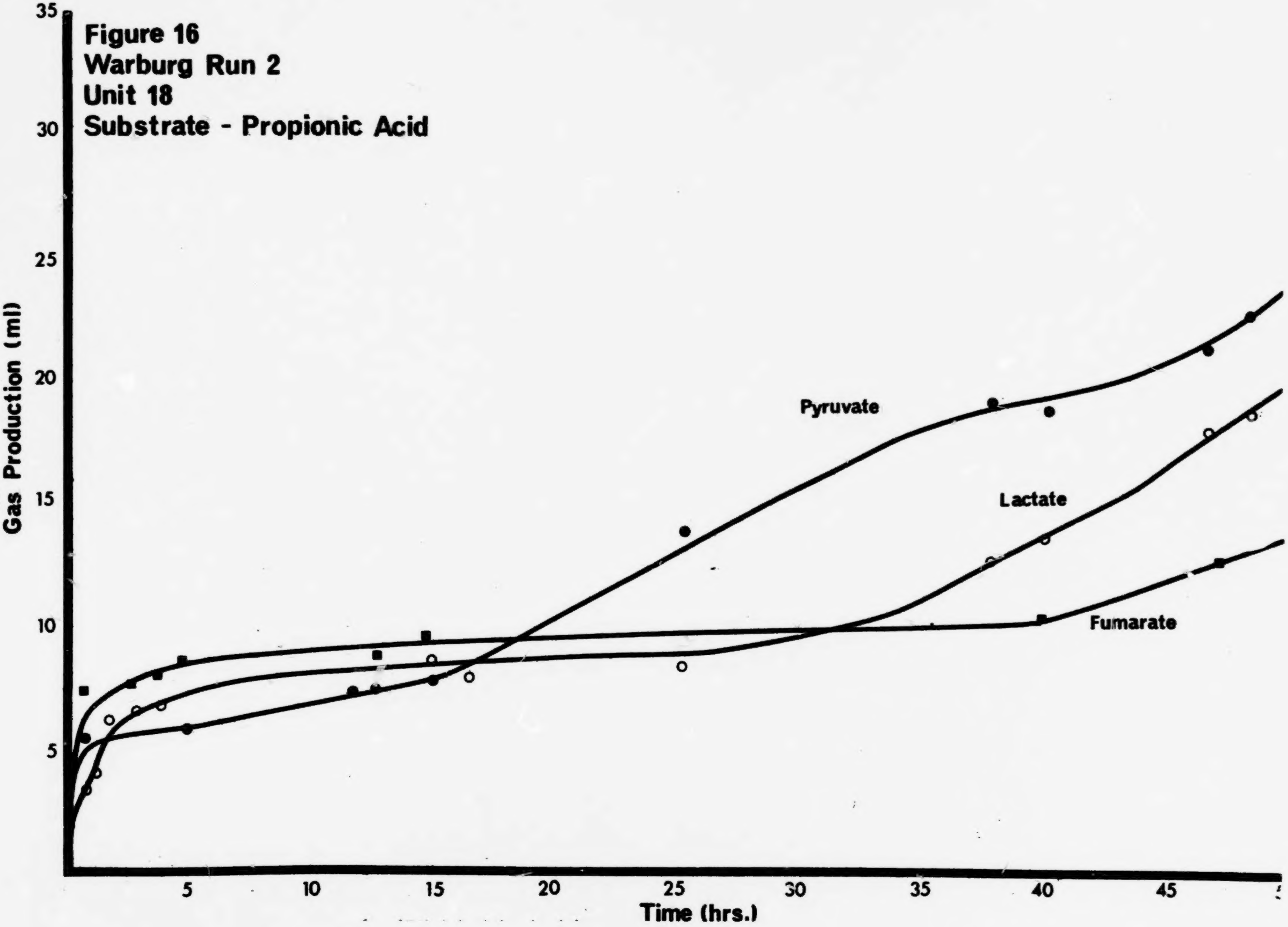


Figure 16
Warburg Run 2
Unit 18
Substrate - Propionic Acid

during the run. Succinic and lactic acids followed closely the gas production of the control indicating that they were not utilized during the 50 hour run. As expected acetic and propionic acids were very efficiently utilized within 24 hours. Based on the theoretical gas yield possible from the amount of these acids fed, 105 and 92 percent of the theoretical value was obtained for acetic and propionic acid respectively.

b) Warburg Run 2

In Figure 16, the amount of gas produced from the utilization of pyruvic, lactic and fumaric acids is plotted. The initial two hour gas production was caused by an acid-alkalinity reaction and cannot be considered as substrate utilization. The plot indicates a slow but fairly constant utilization of pyruvic acid and an eventual acclimation and utilization of lactic and fumaric acids. Unfortunately, the chemical analyses data for the run were misplaced and computations to indicate percent substrate utilization were not made.

2. Succinic Acid - Runs 3 and 4

Two Warburg runs were made using succinic acid acclimated sludge liquor. The gas production data is given in Figures 17 and 18. Run 3 shows a fairly constant gas production in the propionic acid fed unit indicating good substrate utilization. Succinate was not utilized as rapidly as the propionate and shows a fairly long ten hour plateau after the initial gas production. Pyruvic and acetic acids indicate some substrate utilization after an initial lag, however, due to leaks developing in these units, the runs were terminated in less than 25 hours.

A second run using succinic acid was made in order to resolve some of the difficulties encountered during the first run. Numerous analyses were conducted and the results of these are given in the Appendix. Figure 18 showing the gas production data indicates that succinic and acetic acids were readily and efficiently metabolized. Contrary to the first run the gas production data for propionic acid was far below the theoretical yield expected, although the COD data indicates satisfactory utilization. The

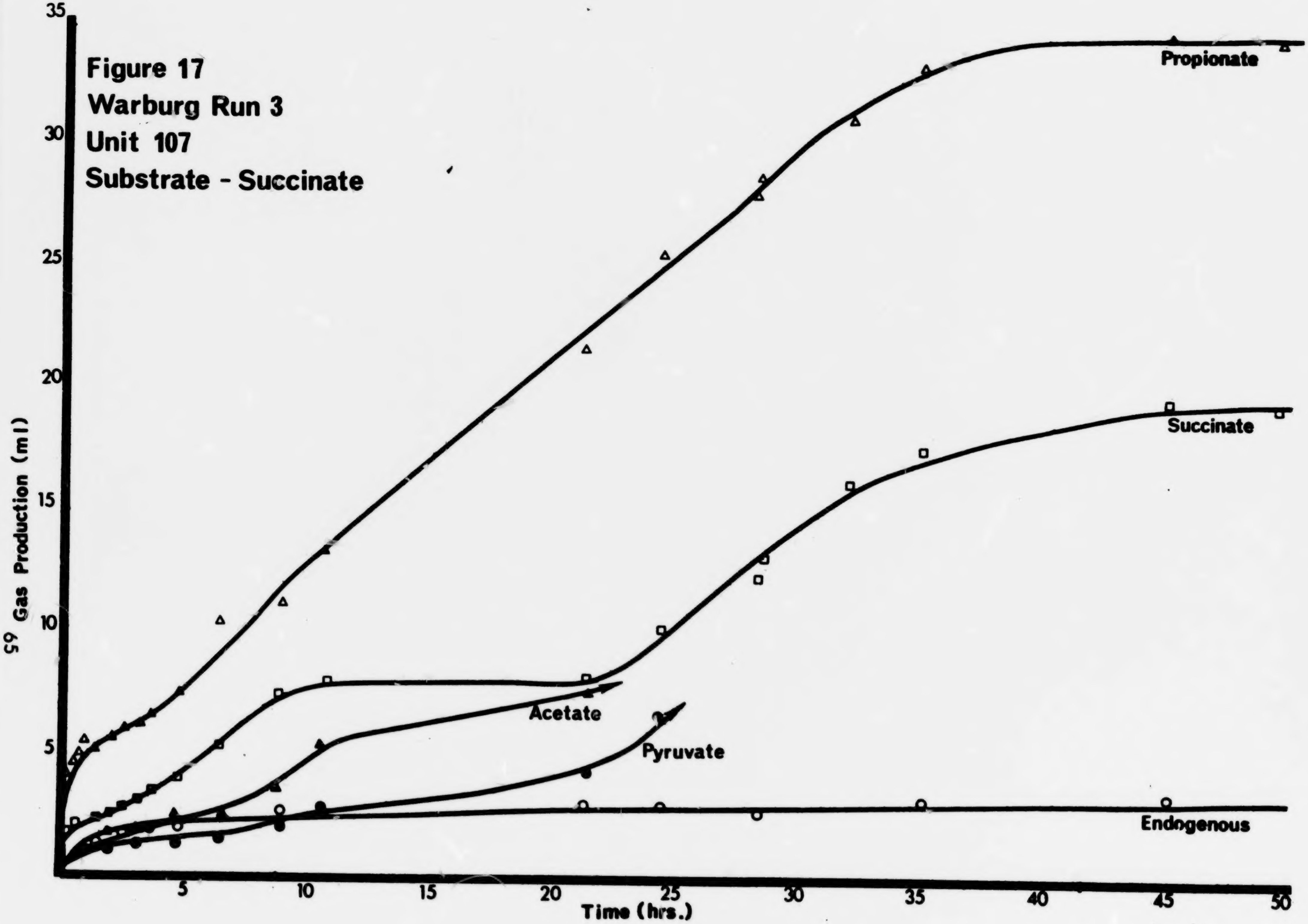


Figure 17
Warburg Run 3
Unit 107
Substrate - Succinate

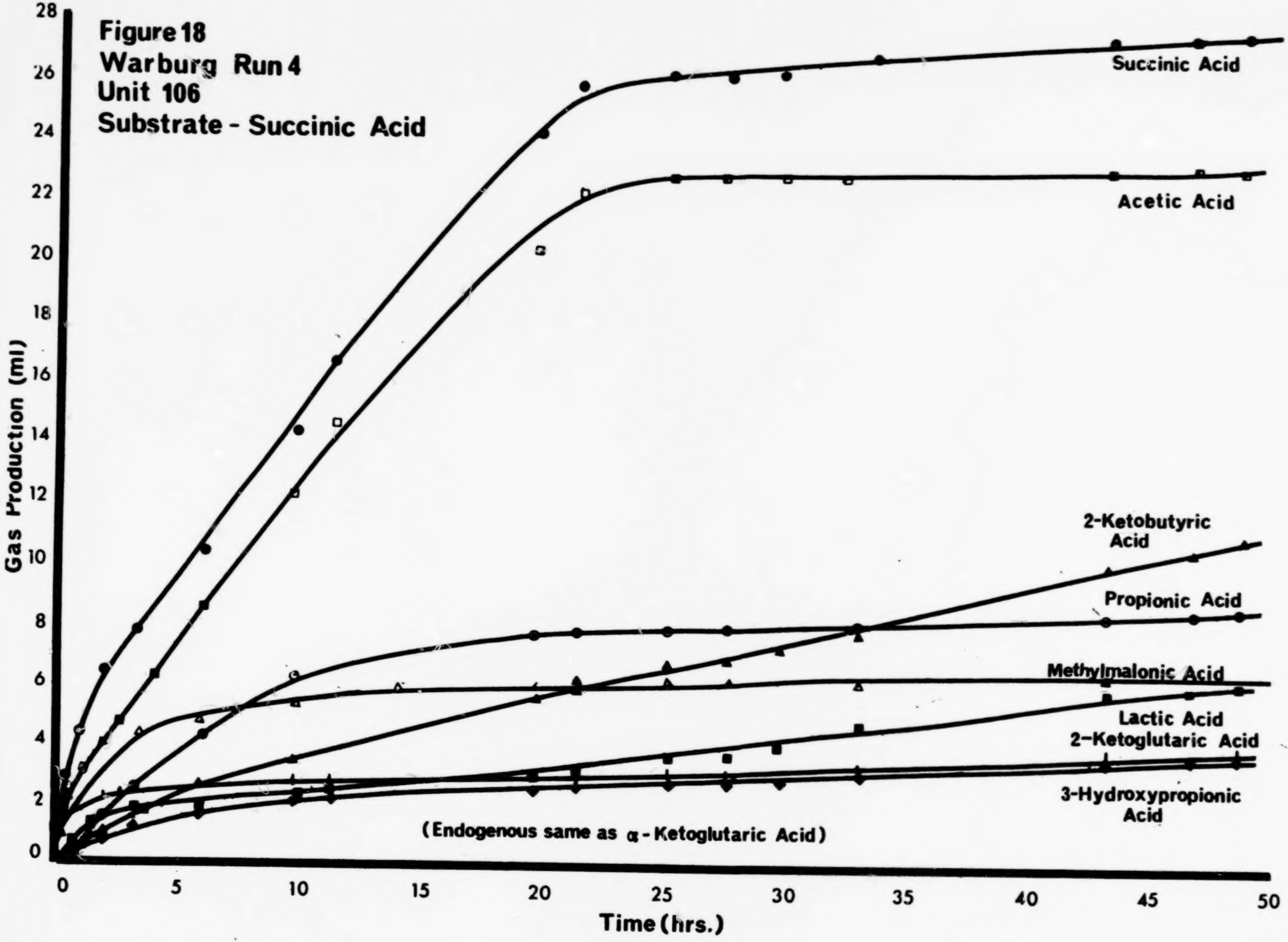


Figure 18
Warburg Run 4
Unit 106
Substrate - Succinic Acid

reason for this apparent contradiction is unknown and may be due to experimental errors. It is also evident that lactic, methylmalonic, 2-ketobutyric, 3-hydroxypropionic and α-ketoglutaric acids were also not metabolized extensively.

3. Lactic Acid - Runs 5, 6, and 7

A total of three Warburg runs were made using liquor from the anaerobic digesters which were acclimated to lactic acid. Results of this study are presented in Figures 19, 20, and 21 and in Tables of the Appendix. As the results vary considerably each run is discussed separately.

a) Warburg Run 5

The gas production for the two unfed controls was 3.1 and 2.5 ml. The average gas production was 2.8 ml and this value was used to correct the gas production for the other units.

The COD reduction for all units varied from about 39 to more than 100 percent, indicating that some substrates could be utilized very well by the microorganisms present in the lactic acid acclimated sludge. Pyruvic acid, ethyl alcohol, formic, acetic and propionic acids which all gave close to 100 percent COD reduction show they are easily fermented by lactic acid acclimated sludge. The ratio of the corrected gas production to the theoretically computed gas production as given in the Appendix tables ranged from 30 to 126 percent with the lower value corresponding to the units giving low COD reduction. Generally this ratio was in agreement with the COD reductions of each individual substrate. The results indicate that pyruvic, acetic, and propionic acids are most likely the main intermediates involved in the lactic acid fermentations. The cumulative gas production is given in Figure 19 and shows that lactic acid sludge can ferment acetic and pyruvic acids at slightly higher rates than it can ferment propionic acid during the initial 18 hours. The rate of gas production for propionic acid was almost constant during the initial 25 hours. Formic acid which most likely is utilized by every sludge adapted to a higher acid substrate was fermented by this lactic acid sludge; it might be an important

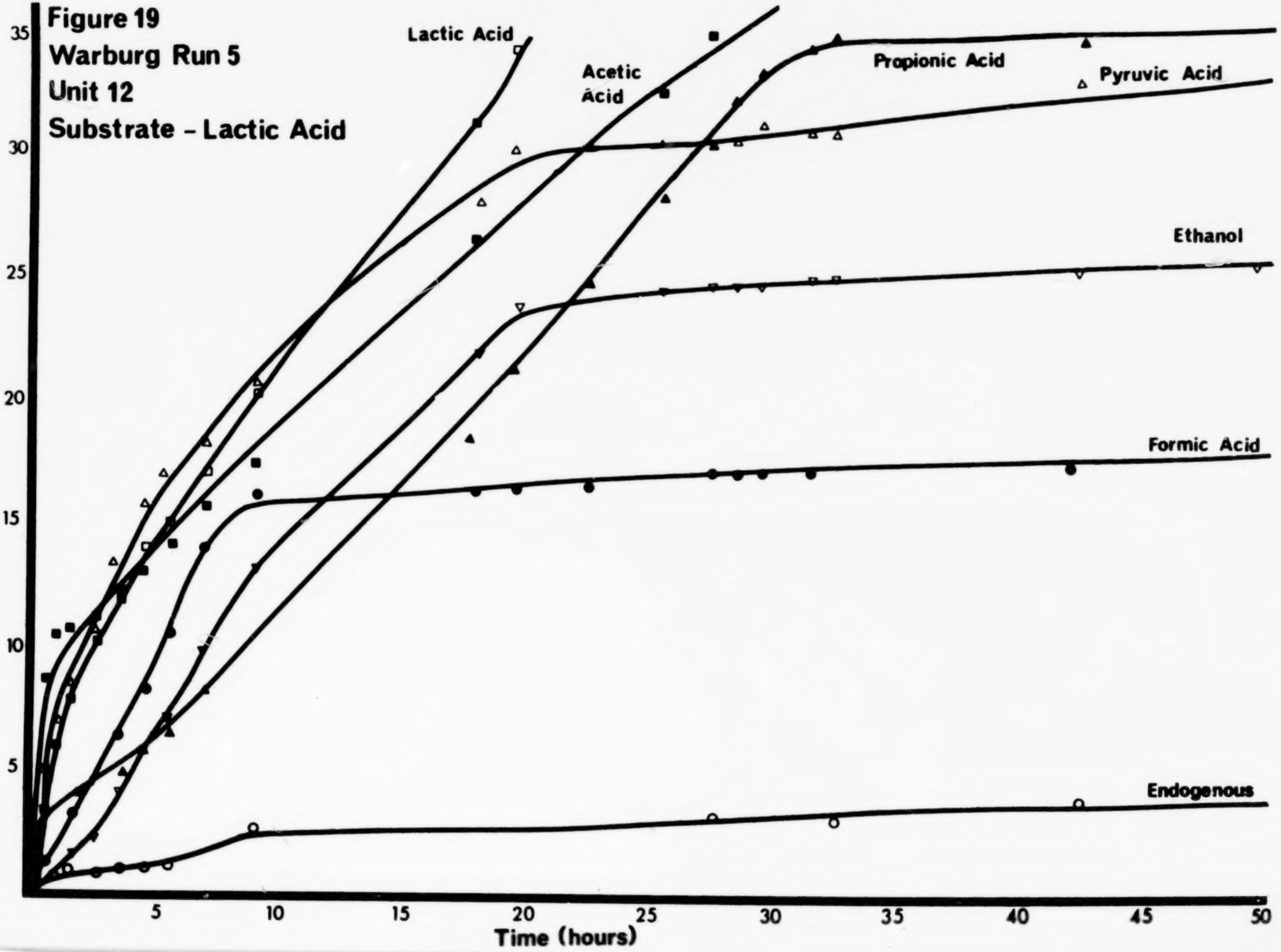


Figure 19
Warburg Run 5
Unit 12
Substrate - Lactic Acid

intermediate in the methane fermentation of substrates and is readily fermented under most conditions. As the results show, ethanol can also be used for lactic acid sludge, but it seems unlikely that it would be an intermediate.

b) Warburg Run 6

As the results show in the Appendix tables and by the gas production as plotted in Figure 20, it is evident that lactic acid sludge can degrade not only lactic acid, but also butyrate, ethyl alcohol, acetaldehyde with an average efficiency above 90% as measured from both COD reduction and the ratio of actual gas production to the theoretical gas production. Also, α-ketoglutarate and glyceraldehyde were metabolized with COD utilization efficiency of 70 percent or greater. In this study malonate was introduced as a substrate in order to see if a 4-carbon intermediate was involved in the lactic acid fermentation. The result showed an average COD reduction of only 42 percent and an average of about 42 percent of the actual gas production to the theoretical gas production if 100 percent of the substrate COD were removed. As most of the gas production for malonic acid occurred during the first two hours of the run, it was not plotted.

c) Warburg Run 7

The results of this run are shown in the Appendix tables and Figure 21. In this run, the four-carbon compounds, succinate and malonate, were included as substrates. The COD reduction and gas production again were very low. For the succinate the COD reduction was one percent and the average ratio of actual gas production to theoretical gas production only 20 percent. For the malonate unit, the COD reduction was only 5 percent and the ratio of actual gas production to the theoretical value was about 21 percent. Again, glyceraldehyde and propionic acid were metabolized as in the previous runs.

4. Glutamic Acid - Runs 8 and 9

Two experiments using the Warburg apparatus were conducted using glutamic acid acclimated sludge liquor from the laboratory

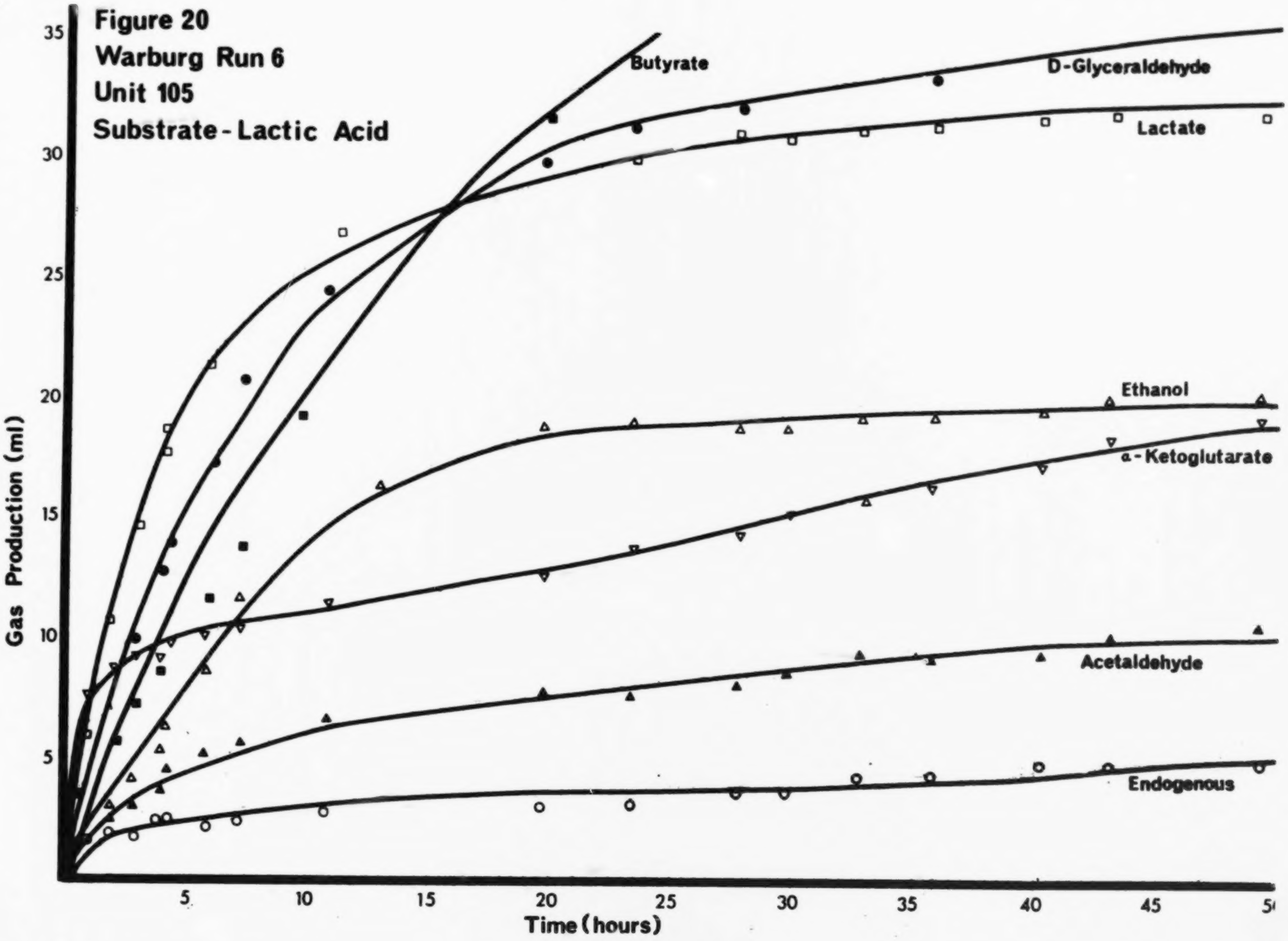


Figure 20
Warburg Run 6
Unit 105
Substrate - Lactic Acid

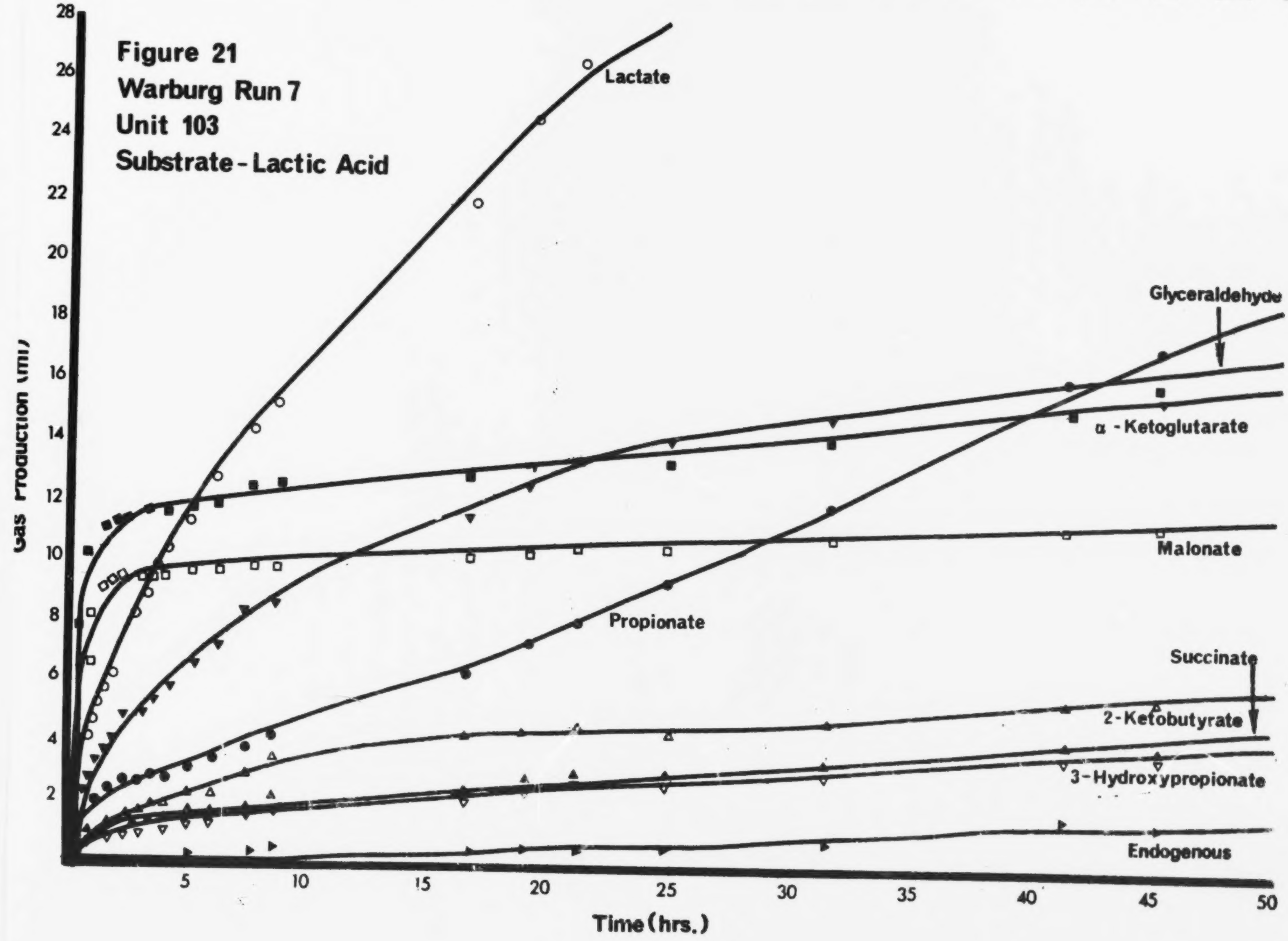


Figure 21
Warburg Run 7
Unit 103
Substrate - Lactic Acid

digesters. Duplicate runs were conducted in this study and are presented as Figures 22 and 23 and in the Appendix tables. Because the results vary considerably in each run, each is discussed separately.

a) Warburg Run 8

The gas production for the two unfed controls were 2.2 ml and 4.0 ml. The average gas production for the controls was 3.1 ml and this value was used to correct the gas production for all the other units. The corrected gas production for all units was confined to a range of 2.6 to 29.3 ml.

The COD reduction for all units varied from 19 to 97 percent, indicating that some substrates could be utilized very well by the microorganisms present in the L(+)-glutamic acid digesters and that these substrates might be intermediates in the degradation of L(+)-glutamic acid.

The experimental results of the ratio of the corrected gas production to the theoretically computed gas production ranged from 11.3 percent to infinity with two ratios greater than 100 percent, and were probably due to the techniques employed. Although the results of the chemical analyses were not excellent, the ratios are useful in interpreting some of the C-14 tracer studies. The high substrate utilization ratios indicated that the corresponding substrates could be degraded by the microorganisms present in the sludge liquor of the L(+)-glutamic acid digesters and that these substrates might be intermediates.

From the results shown in the Appendix table it can be seen that formic, pyruvic and acetic acids are probably intermediates in the degradation of L(+)-glutamic acid.

b) Warburg Run 9

The gas production for the two unfed controls were all zero while the corrected gas production varied from zero to 43.6 ml for all other units.

The COD reduction for all units varied from 7.0 to 101.2 percent while the experimental results of the ratio of the corrected gas production to the theoretical gas production for

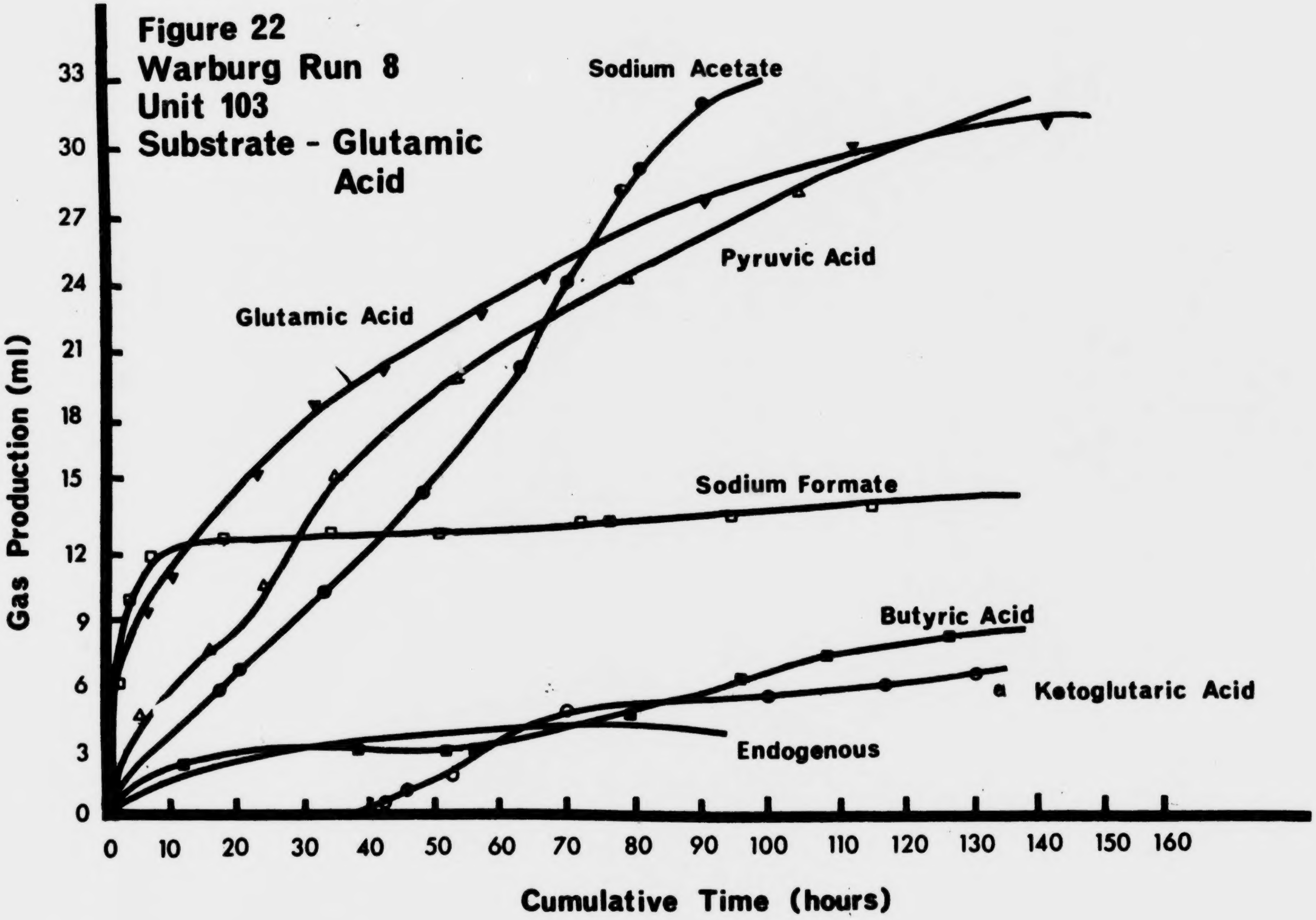


Figure 22
Warburg Run 8
Unit 103
Substrate - Glutamic Acid

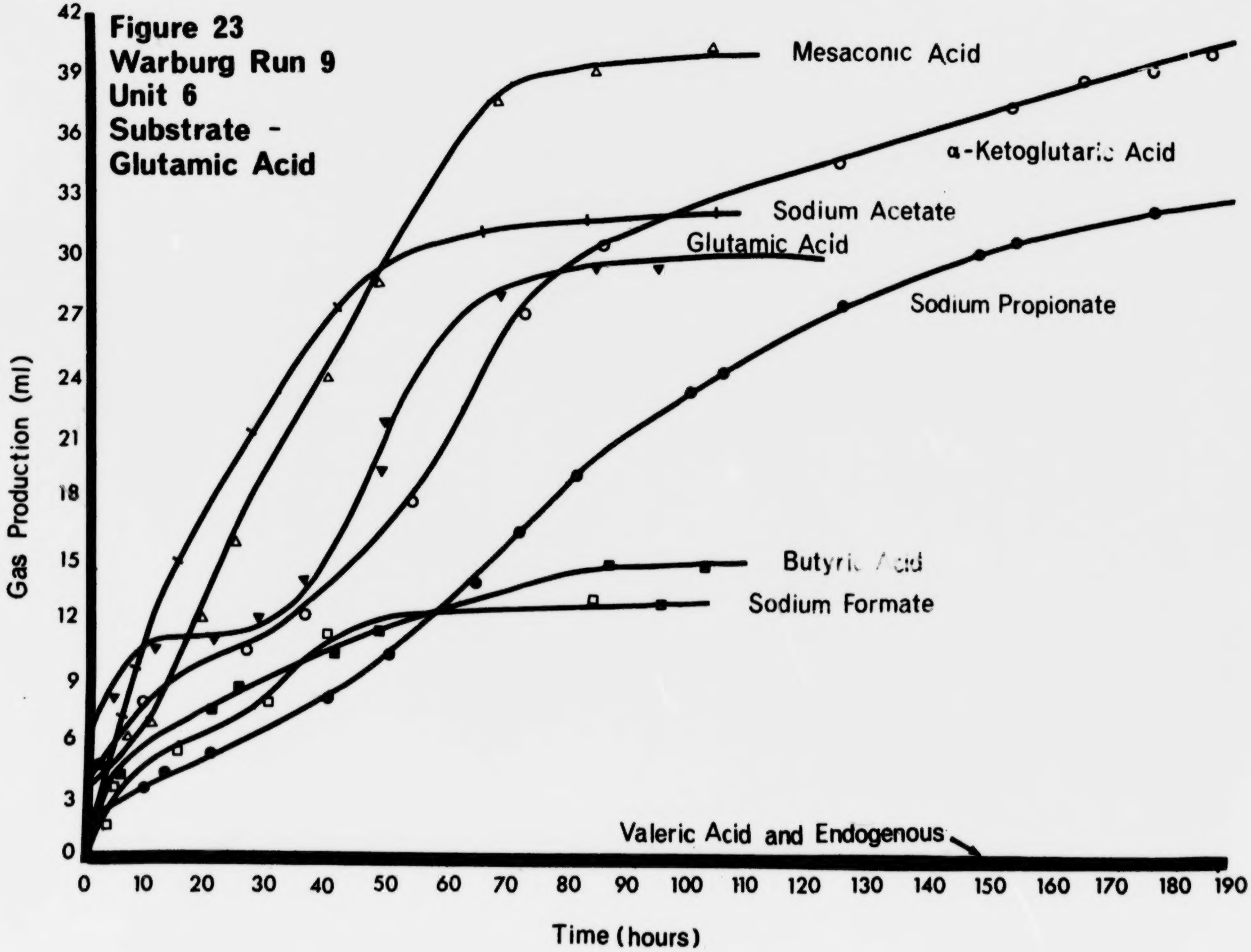


Figure 23
Warburg Run 9
Unit 6
Substrate -
Glutamic Acid

100 percent COD reduction ranged from zero percent to infinity with six ratios greater than 100 percent. From the results shown in the Appendix, it can be seen that formic, acetic, propionic, α-ketoglutaric and mesaconic acids may be intermediates in the degradation of L(+)-glutamic acid.

VI DISCUSSION

A. Propionic Acid

Previous tracer work on this major intermediate of the methane fermentation indicated vastly different results. Stadtman and Barker (33) using a purified culture of methane bacteria showed that the number three carbon of propionic acid formed methane primarily. In the work of Buswell et al (34) however, it was reported that randomization of the number three carbon of propionic acid probably occurred through a four carbon intermediate resulting in a significant yield of carbon dioxide from the number three carbon.

During the current investigation the use of propionic acid-3C-14 gave experimental ratios of activity of carbon dioxide to methane ranging from 0.03 to 0.83. The lower ratios compare very favorably to the results obtained by Stadtman and Barker (33) represented in Figure 3. The higher ratios can be explained on the basis of a mixed population of bacteria in which some or one of the species are capable of converting the number three carbon of propionic acid to methane while others randomize this carbon through a four carbon intermediate as proposed by Buswell et al (34) and a pathway has been postulated as presented in Figure 4. Another possible explanation, especially in the run where a specific activity ratio of 0.83 was obtained, is an alternate pathway of the type reported in animal tissues and in Clostridium kluyveri (42, 43). The pathway involves the beta-oxidation of propionic acid and the subsequent oxidation of the beta or number three carbon. Such a pathway would yield labeled carbon dioxide from propionate-3C-14 or a specific activity ratio of carbon dioxide to methane 3.0. Thus, a combination of two or all three of these pathways could explain the results obtained using these laboratory digesters which had been acclimated to propionic acid for a long period of time. In other studies in which two digester units were fed raw sludge daily, propionic acid-3C-14 was added. In both of these studies the radioactivity was equally distributed between the carbon dioxide and methane indicating that beta-oxidation of propionate may be more important in digesters fed raw sludge.

When using propionic acid-2C-14, the experimental ratios of the activity of carbon dioxide to methane were 1.25 and 1.34. These two values lie between the theoretical results predicted in Figures 3, 4 and by beta-oxidation giving evidence that all of these pathways or another may be involved as degradative mechanisms.

Other studies using carbon-14 labeled sodium bicarbonate with the propionic acid substrate gave specific activity ratios of carbon dioxide to methane slightly below those predicted in the theoretical considerations but appear to be within reason. The bicarbonate studies also indicate that one mole of acetate is produced from each mole of propionic acid that is metabolized. In the studies where butyric acid-2C-14 was used, the ratios of the specific activity of carbon dioxide to methane extended from 1.08 to 1.39 indicating that beta-oxidation was not a primary mechanism in its degradation.

From the identification of volatile acids by hydrogen flame chromatography, it was found that acetic and propionic acids were the acids always present in the greatest concentrations. Butyric acid was also found but at low concentrations in 45 of the 125 samples analyzed. Isobutyric and valeric acids were found in only two of the samples analyzed. Since acetic and propionic acids are present as a feed or intermediates in the pathways discussed these results were in agreement with those obtained in the C-14 tracer studies. The small concentration of butyric acid could arise from condensation of two molecules of acetic acid as has been reported by Barker (4).

Warburg experiments using sludge liquor acclimated to propionic acid indicate that acetate, lactate, pyruvate and fumarate were utilized with varying efficiencies. The rapid uptake of acetate confirms the volatile acid studies which indicated that acetic acid was a major intermediate present. The less readily metabolized lactic, pyruvic and fumaric acids could be intermediates in the conversion of propionic acid to a four carbon compound but this would be purely speculation. Although succinic acid was not metabolized, this does not necessarily preclude its participation as an intermediate. It may very well be an intracellular intermediate which cannot pass through the cell wall.

The results of the propionic acid experiments generally confirm the work of Buswell et al (34) and Stadtman and Barker (33) showing that the number three carbon may or may not yield methane. Although the exact mechanism has not been determined a third mechanism involving beta-oxidation of propionate has also been proposed as a participant. It is possible that two or three of the mechanisms discussed or perhaps others may also be important. Thus, the degradation of propionic acid does not appear to be limited primarily to one mechanism in most cases where anaerobic digestion is used.

B. Succinic Acid

Succinic acid was studied since it is a very important compound in intermediary metabolism. Also, it was believed that it would be able to help answer some of the questions involving the questions concerning the number three carbon of propionic acid.

Using succinic acid-2C-14, the specific activity of carbon dioxide to methane extended from 1.0 to 1.36 in four separate runs which is well above the ratio predicted in the pathway presented in Figure 5. Thus, another pathway which allows a greater conversion of the number two carbon of succinate to carbon dioxide must also be involved in the degradation. A conversion of a portion of the succinate to carbon dioxide through the kreb cycle could explain the phenomenon. Oxygen however, may be required but was not present.

Results from the use of radioactively labeled sodium bicarbonate were in fairly good agreement with the postulated pathway given in Figure 5. These results indicate that 57 percent of the methane produced originates from acetic acid degradation and the remainder from carbon dioxide reduction. In effect from each mole of succinic acid metabolized one mole of propionic acid would be formed. As acetic acid is a natural breakdown product of propionic acid one mole of acetic acid would also be produced for each mole of succinic acid degraded.

Volatile acid studies performed on the succinic acid units confirmed that the major acid produced was propionic with a smaller amount of acetic also present. Further confirmation of the importance of acetic and propionic acids resulted from Warburg runs in which both compounds were readily metabolized. Propionic and acetic acids, then, may be considered as major intermediates in the degradation of succinic acid. The presence of large amounts of propionic acid in the sludge liquor of succinic acid units may indicate a close relationship between the propionic and succinic acid degradative mechanisms. It is conceivable that the same species of methane organisms exist in both units in which case succinic acid would most likely be utilized by non-methane producing organisms present; if this assumption is correct, then the propionic acid found may be metabolized by a mechanism similar to that for propionic acid degradation. The acetic acid present would then be the result of the further degradation of propionic acid. More information could be obtained by conducting "pulse" experiments and then trapping the propionic and acetic acids found. The distribution of the label would give some indication of the breakdown of succinate.

A striking result of this run was the utilization of propionate. The importance of propionic acid is also indicated in the volatile acid studies where it was shown to be the major volatile acid present.

C. Lactic Acid

In the carbon tracer studies on lactic acid, use was made of carbon-14 labeled lactic acid in the numbers, one, two and three positions and $NaHC^{14}O_3$. Labeled sodium bicarbonate was used to determine the quantity of methane derived directly from the reduction of carbon dioxide. The results showed that slightly more than one-third of the methane was derived from reduction of carbon dioxide, while almost two-thirds was derived from the methyl group of acetic acid, an intermediate in the degradation. Similar results were obtained using lactic acid-1C-14 indicating that the carboxyl carbon was decarboxylated and can be considered to be synonymous with carbon dioxide or bicarbonate.

The determination of whether the "by-pass succinic acid pathway" or some other symmetrical four-carbon compound was involved in the lactic acid fermentation would be more readily indicated using 2 or 3C-14 labeled lactic acid. If randomization occurred a symmetrical four-carbon compound would lead to the appearance of C-14 equally distributed in the α and β positions of a three-carbon intermediate such as propionic acid.

One run using lactic acid-2C-14 gave a specific activity ratio of carbon dioxide to methane of 1.14 which is close to the predicted value of 1.0 (Figure 6), assuming the four-carbon intermediate. If it was assumed that lactic acid was directly reduced to propionate without the participation of the symmetrical four-carbon intermediate, the theoretical predicted specific activity ratio of carbon dioxide to methane would be 3.0 which is much higher than the actual result obtained.

Five runs using radioactively labeled lactic acid on carbon number three gave specific acitivity ratios of 1.46, 1.18, 1.0, 1.06 and 0.32. As only one of the runs compared favorably to the theoretically predicted value of 0.33, two or more pathways may have been involved. If propionate was directly derived from the dehydrogenation of lactic acid, the third carbon of lactate should remain intact, if alpha-oxidation was the prime degradation mechanism. If this were true, using lactic acid-3C-14 the specific activity ratio of carbon dioxide to methane should be equal to zero and would imply that the radioactivity was derived entirely from methane. As the experimental results indicated that in four of the five runs slightly more than half the radioactivity was in the carbon dioxide, this mechanism does not appear to be very important. Another mechanism may be involved which allows the methyl carbon of lactic acid to be converted to carbon dioxide. Most likely, as in the propionic and succinic acid studies two or more mechanisms, such as the beta-oxidation of propionic acid, participate in the lactic acid degradation.

In the volatile acids identification study, propionic and acetic acids predominated, with the occasional appearance of small amounts of butyric and valeric acids. Since the concentration of the higher acids was small they were considered to be minor acids. They usually appeared following the slug feeding of substrate and especially when the concentration of propionic and acetic acids increased. They were probably formed through condensation reactions involving acetic and propionic acids. These minor acids would be converted to propionic and acetic acids as the concentration of the latter acids progressively decreased.

The results of the Warburg studies showed that lactic acid sludge could ferment not only lactic acid but also; ethanol, acetaldehyde, propionic, pyruvic, acetic, α-ketoglutaric and butyric acids, while it failed to ferment succinic acid and only partial degradation was obtained with malonic acid. Since propionic and acetic acids were also found consistantly in the volatile acids study, there is no doubt that they are important intermediates. Pyruvic acid was expected to be degraded by lactic acid sludge, as it is a known intermediate and its configuration is much like that of propionate.

In the pathway proposed in Figure 6, it was reasoned that the "by-pass" four-carbon succinic acid or a similar intermediate was the means by which randomization occurred. Therefore, succinic or malonic acids could be involved. However, the fact that the Warburg results failed to show that lactic acid acclimated sludge could use succinic and malonic acids readily does not disprove the possibility of their being intermediates. It may be due to the inability of the succinic or malonic acid molecule to penetrate the cell wall of the lactic acid acclimated organisms. It could also be due to the absence or lack of extracellular enzymes. Succinic or malonic acids could be intermediates existing inside the cell structure with concentrations so low that they are never detected outside the cell. As discussed, in spite of the Warburg results obtained, succinic or malonic acids could be intermediates in this fermentation.

The similarity of the various intermediates and possible pathways involved in the degradation of lactic, propionic and succinic acids suggests possible cyclic and interconvertible reactions. These many possibilities add to the difficulty of interpreting the results and to the cause of the preferential conversion of the number three carbons of lactate and propionate and the number two carbon of succinate to carbon dioxide. A further consideration is the apparent dynamic nature of the biological populations participating in the degradative reactions. The significant differences in specific activity ratios of carbon dioxide to methane using similar substrates indicate that changing populations play an important part in the metabolism of these compounds.

Unfortunately, the biological mass was not measured during these investigations and the effect of synthesis cannot be determined. With propionic acid, synthesis would most likely be lower than would be expected with lactic or succinic acids; but it is obvious that the greater the synthesis the greater would be the incorporation of labeled carbon into cell mass. With the typical intracellular reactions occurring and cell material utilization greater quantities of labeled carbon dioxide would result. Thus the greater the synthesis the greater would be the labeling of carbon dioxide.

D. Glutamic Acid

In the study of L(+)-glutamic acid, use was made of C-14 in sodium bicarbonate and the number one, two and five carbon positions of glutamic acid. According to the pathway shown in Figure 7, with the number two carbon of glutamic acid labeled only the methane of the gaseous fraction should become labeled. As the ratios of the specific activity of carbon dioxide to methane were 0.19 and 0.12 the postulated pathway appears to be valid. The small amount of labeled carbon dioxide obtained was most likely caused by synthesis turnover, or, to a lesser degree the possibility of carbon dioxide fixation.

While using glutamic acid, labeled in either of the carboxyl groups, or labeled sodium bicarbonate, theory would predict similar results if rapid decarboxylation occurred randomizing the carbon dioxide produced with the alkalinity and gaseous fraction present in the digester. Accordingly, from each mole of glutamic acid fermented, one-fourth mole of carbon dioxide would be reduced to methane and two moles of acetic acid formed. As two moles of methane would be produced from the two moles of acetic acid, one out of every nine moles of methane produced would come from carbon dioxide reduction and the remainder from acetic acid degradation. Of the eight laboratory digesters operated to confirm the theory, seven were within ten percent of the theoretical specific activity ratio and the eighth within 20 percent.

From the identification of volatile acids by hydrogen flame chromatography, it was determined that acetic acid was always the major acid present in the glutamic acid fermentation. Although propionic acid was very often identified, it was not as prevalent in solution as the acetic acid. Because propionic acid yields acetic acid in the degradation to the end products, carbon dioxide and methane, the overall importance of acetic acid is further amplified. No higher volatile acid was found in this fermentation; thus, these results were in agreement with the theory presented.

Results from the anaerobic Warburg runs indicate good utilization of the following compounds: formic, acetic, propionic, pyruvic, α-ketoglutaric and mesaconic acids. Of these compounds only α-ketoglutaric acid is not postulated to be an intermediate in the degradation of glutamic acid. This closely related compound has an alpha keto group substituted for the alpha amino group. These results tend to confirm the postulated pathway to a greater extend but utilization of the compounds by the microbial population present does not necessarily indicate that they are intermediates.

Although some of the chemical analyses for the Warburg runs were semi-quantitative due to the size of the sample available, the results of these studies aid in the interpretation of some of the C-14 work. Further, the excellent utilization of acetate and propionate is also indicated by the fact that these two acids were the only volatile acids found in glutamic acid acclimated sludge liquor.

Since the suggested metabolic scheme goes thru propionic acid other possibilities would appear possible such as occurred in the other studies. However, in the glutamic acid runs, with the labeled compounds used, wide variations did not occur.

On the whole, the results of the carbon tracer experiments, volatile acid identification, and Warburg runs confirm the biochemical pathway proposed in Figure 7.

E. Oleic Acid

In the study to determine the degradative pathway for oleic acid, the use of C-14 labeled sodium bicarbonate and oleic acid-1C-14 was made. The experimental results of the ratio of the specific activity of carbon dioxide to methane were 4.08, 3.48, 3.49 and 3.00. The first three values compared well to the predicted results for the pathway postulated in Figure 8 but differed from the pathway shown in Figure 9. The last value was approximately the arithmetic mean of the two theoretical results which were 2.68 and 3.40, indicating that both pathways may have been involved as degradative mechanisms in this study.

From the identification of volatile acids by hydrogen flame chromatography, it was found that acetic acid was the acid always present in the greatest concentration. Propionic acid was found in trace amounts in 5 of the 24 samples analyzed and no higher volatile acid was found in any of the samples tested. No propionic acid was found in the sludge samples from the digester units which had ratios of the specific activity of carbon dioxide to methane of 4.08, 3.48 and 3.49 while propionic acid was found present in the sludge liquor from the digester unit which had a specific activity ratio of carbon dioxide to methane of 3.00. Since acetic acid and propionic acid are both present in the pathway shown in Figure 8, the acetic acid is the only volatile acid present in the pathway shown in Figure 9, these results agree with the results obtained in the tracer studies. It appears likely that oleic acid was primarily degraded by beta-oxidation through the pathway shown in Figure 8 and that the pathway shown in Figure 9 played a secondary role.

F. Benzoic Acid

The results of the carbon tracer work compare favorably with the work of Fina and Fiskin (26) and are shown in Table 8 and Figure 10. As previously indicated the number one carbon was almost completely converted to methane and only a small amount of the tracer fed was recovered in the non-methane fraction. Labeled bicarbonate showed the extend of reduction of carbon dioxide to methane of exogenously added carbon dioxide and indicated that one mole of methane was labeled for every five moles of carbon dioxide. As 80 percent of the methane produced arose from acetic acid utilization, each mole of benzoic acid must yield three moles of acetic acid. The results using benzoic acid-7C-14 should be similar to those for $NaHC^{14}O_3$ with a subtle difference. Assuming that decarboxylation occurs, the preferencial position of radioactive carbon dioxide will be within the cell. The assumption is based on the benzoic acid decarboxylation reaction which is most likely carried out by a non-methane organism and the labeled carbon dioxide would not be immediately available for reduction to methane. As the study used a one-time slug feed of the labeled compound the final specific activity ratio of carbon dioxide to methane should then indicate slightly more activity in the methane fraction when compared to the fraction obtained in the bicarbonate study. The experimental results substantiate this interpretation.

The results show that an aromatic ring may be degraded anaerobically with good efficiencies by the bacterial species present in the sludge liquor. It has also been shown that the carboxyl carbon is almost equivalent to exogenously added carbon dioxide, since with either labeled carboxyl of exogenous carbon dioxide, the specific activity ratio is about the same. In addition, the point of ring cleavage must occur between the numbers 1 and 6 or 1 and 2 carbons.

To differentiate between these points of cleavage it would be necessary to stop the reaction before completion and to isolate the intermediate compounds. It is also most likely that the organisms responsible for ring cleavage are not methane organisms but some of the facultative or anaerobic organisms present in the sludge liquor. The organisms would, therefore, have to produce an intermediate such as acetic acid that would be degraded by the methane organisms.

VII CONCLUSIONS

1. Three pathways may be involved in the degradation of propionic acid. Alpha and beta oxidation may be involved or a third mechanism involving a randomization of the number three carbon, possibly through a four-carbon intermediate.

2. Propionic and acetic acids are major intermediates in the degradation of succinic and lactic acids. Several pathways appear to be involved including alpha and beta oxidation of the intermediate propionic acid.

3. Glutamic acid was probably degraded to methane and carbon dioxide through mesaconic, citramalic, pyruvic, lactic, propionic and acetic acids. Essentially two moles of acetic acid were produced for each mole of glutamic acid metabolized.

4. In the oleic acid fermentation, beta-oxidation appeared to be the major mechanism. From each mole of oleic acid degraded, essentially nine moles of acetic were produced before the final end products of methane and carbon dioxide were formed.

5. The fermentation of benzoic acid gave three moles of acetic acid. The number one carbon yields essentially 100 percent methane.

6. Acetic acid was the major volatile acid intermediate encountered in most of the methane fermentation studies. Propionic acid was second in importance.

BIBLIOGRAPHY

1. Heukelekian, H. and Heinemann, B., "Studies on the Methane-Producing Bacteria. III. Digestion of Sewage Solids by the Addition of Enriched Culture of Methane-Producing Organisms", Sewage Works Journal, 11, 571-585 (1939).

2. Buswell, A.M. and Neave, S.L., "Laboratory Studies of Sludge Digestion," Illinois Division of State Water Survey, Bulletin No. 30 (1930).

3. Pine, M.J. and Barker, H.A., "Studies on the Methane Fermentation XII. The Pathway of Hydrogen in the Acetate Fermentation," Journal of Bacteriology, 71, 644 (1956).

4. Barker, H.A., Bacterial Fermentations, John Wiley and Sons, Inc., New York (1956).

5. Stadtman, T.C. and Barker, H.A., "Studies on the Methane Fermentation IX. The Origin of Methane in the Acetate and Methanol Fermentations by Methanosarcina," Journal of Bacteriology, 61, 81-86 (1951).

6. Stephenson, M. and Strickland, L.H., "III. The Bacterial Formation of CH_4 by the Reduction of One-Carbon Compounds by Molecular H_2," Biochemical Journal, 27, 1517 (1933).

7. Kluyver, A.J. and Schnellen, G.T.P., "On the Fermentation of Carbon Monoxide by Pure Cultures of Methane Bacteria." Archives of Biochemistry, 14, 57-70 (1947).

8. Pine, M.J., "Methane Fermentation of Formate by Methanobacillus Omelianskii" Journal of Bacteriology, 75, 356 (1958).

9. Bryant, M.P., Wolin, E.A., Wolin, M.J., Wolfe, R.S., Mandel, M., "Methanobacillus Omelianskii, a Symbiotic Association of Two Bacterial Species, Bacteriological Proceedings A109, p. 19 (1967).

10. Tarvin, D. and Buswell, A.M., "The Methane Fermentation of Organic Acids and Carbohydrates," Journal of American Chemical Society, 56, 1751-1775 (1934).

11. Buswell, A.M. and Hatfield, W.D., "Anaerobic Fermentations," Illinois State Water Survey, Bulletin No. 32 (1939).

12. Flavin, M., Ortiz, P.M. and Ochoa, S. 1955-1957 Nature 176; 823-826 (1955).

13. Lardy, H.A. and Adler, J., "Synthesis of Succinate from Propionate and Bicarbonate by Soluble Enzymes from Liver Mitochondria," Journal Biological Chemistry, 219, 933 (1956).

14. Phares, E.F., Delwiche, E.A. and Carson, S.F., "Succinic Acid Decarboxylation System in Propionibacterium Pentosaceum and Veillonella Gazogenes, II. Evidence for an Active "C" Complex", *J. Bact.*, 71, 604-610, (1956).

15. Delwiche, E.A., "Mechanism of Propionic Acid Formation by Propionibacterium Pentosaceum", *J. Bact.*, 56, 811-819 (1948).

16. Wood, H.G., Stjernholm, R. and Leaver, F.W., "The Role of Succinate as a Precursor of Proprionate in the Propionic Acid Fermentation," *J. Bacteriology* 72, 142 (1956).

17. Jeris, J.S. and McCarty, P.L., "Biochemistry of Methane Fermentation Using C^{14} Tracers", Proceedings 17th Annual Purdue Industrial Waste Conference, 1962, and Journal Water Pollution Control Federation, February, 1965.

18. Fruton, J.S. and Simmonds, S., General Biochemistry, John Wiley and Sons, Inc., New York (1959).

19. Gortner, R.A. and Gortner, W.A., *Outlines of Biochemistry*, 3rd Edition, John Wiley and Sons, Inc., New York (1950).

20. McKinney, R.E., "*Microbiology for Sanitary Engineers*", McGraw-Hill Book Company, Inc., New York (1962).

21. Symons, G.E. and Buswell, A.M., "The Methane Fermentation of Carbohydrates," *Journal of American Chemical Society*, 55, 2028-2036 (1933).

22. Wachsman, J.T. and Barker, H.A., *Journal of Biological Chemistry*, 217, 695 (1955).

23. Sleeper, B.P., "The Bacterial Oxidation of Aromatic Compounds C^{14}", J. Bact. 62:657 (1951).

24. Parr, W.H., Evans, R.A., and Evans, W.C., "The Mechanism of the Bacterial Oxidation of Certain Aromatic Compounds, Together with the Preparation and Properties of a cell-free enzyme system which accomplishes ring cleavage", Biochem. Jour., 45, Proc. Biochem. Soc., xxix (1949).

25. Harborne, J.B., "Biochemistry of Phenolic Compounds", Academic Press, London, (1964).

26. Fina, L.R. and Fiskin, A.M., "The Anaerobic Decomposition of Genzoic Acid During Methane Fermentation. II. Fate of Carbons One and Seven", *Archives of Biochemistry and Biophysics*, 91, 163 (1960).

27. Standard Methods for the Examination of Water and Wastewater, 12th Edition, American Public Health Association, Inc., New York (1965).

28. Standard Methods for the Examination of Water and Wastewater, 11th Edition, American Public Health Association, Inc., New York (1960).

29. McCarty, P.L., "The Significance of Ion Concentration in the Anaerobic Biochemical Decomposition of Acetate," Ph.D. Thesis, M.I.T.,(1959).

30. Jeris, J.S., "A Rapid COD Test", Water and Wastes Engineering (May, 1967).

31. McCarty, P.L., Jeris, J.S., McKinney, R.E., Reed, K. and Vath, C.A., Microbiology of Anaerobic Digestion, M.I.T. (1962).

32. McCarty, P.L., Jeris, J.S. and Murdoch, W., "Individual Volatile Acids in Anaerobic Treatment", Journal Water Pollution Control Federation, 35, 1501, (1963).

33. Stadtman, T.C. and Barker, H.A., "Studies on the Methane Fermentation. VIII. Tracer Experiments on Fatty Acids Oxidation by Methane Bacteria," Journal of Bacteriology, 61, 67-80 (1951).

34. Buswell, A.M., Fina, L., Mueller, H.A., and Yahiro, A., "Use of C^{14} in Mechanism Studies of Methane Fermentation. II. Propionic Acid," Journal of American Chemical Society, 73, 1809 (1951).

35. Leaver, F.W., Wood, H.G. and Stjernholm, R., "The Fermentation of Three Carbon Substrates by Clostridium propionicum and Propionibacterium", Journal of Bacteriology, 70, 521-530 (1955).

36. Barban, S. and Ajl, S., "Interconversion of Propionate and Succinate by Propionibacterium pentosaceum", J. Biol. Chem., 192, 63 (1951).

37. Gutierrez, J. "Journal of Bacteriology" 66, 123-128 (1953).

38. Fitz, A. 1878-1880. Ber. deut. chem. Ges. 11: 1890-1899 (1878); 12: 474-481 (1879) 13: 1309-1312 (1880).

39. Von Freudenreich, E., and Orla-Jensen, S. 1906 Zentr. Bakt., II 17: 529-546 see also Orla-Jensen, S. Ibid., II 13: 161-170 (1904).

40. Van Niel, C.B. 1928. The Propionic Acid Bacteria. Haarlem, J.W. Baissevain and Co., 187 pp.

41. Johns, A.T. 1951. Journal General Microbiology 5: 326-336, 337-345.

42. Vagelos, P.R. and Earl, J.M., "Propionic Acid Metabolism III β-hydroxypropionyl Coenzyme A and Malonyl Semialdehyde Coenzyme A, Intermediates in Propionate Oxidation by Clostridium Kluyveri", J. Biol. Chem. 234, 2272-2280 (1959).

43. Advances in Enzymology, Vol. 26, Edited by F.F. Nord, Interscience Publishers (1964) Cahpter by Kaziro, Y. and Ochoa, S., "The Metabolism of Propionic Acid".

44. Lange, N.A., Handbook of Chemistry, 8th Edition, Handbood Publishers, Inc. Sandusky, Ohio (1952).

45. Handbook of Chemistry and Physics, 39th Edition, Chemical Rubber Publishing Co., Cleveland, Ohio (1958).

APPENDIX I

A RAPID COD TEST

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A rapid COD test

A RAPID, inexpensive and simple method for COD analysis, which has been used successfully on a wide variety of organic compounds, is described. The method employs a mixture of potassium dichromate in sulfuric and phosphoric acids. Other chemicals used include silver as a catalyst and mercury for the elimination of chloride interference.

The Standard Methods COD test[1] is a valuable tool that is widely employed in the analysis of wastewaters. The method has many desirable features and offers the advantages of manipulative ease, efficiency of oxidation, dependability, and obvious reproducibility. However, a Standard Methods COD test will require approximately 3 hr for completion and, in terms of chemicals, represents a cost of approximately 20 cents per analysis. When the COD test is employed on a routine day-to-day basis for the analysis of wastewaters that do not vary considerably in their composition—as is the case in many control or monitoring processes—this may represent a substantial investment in terms of both labor and capital. The studies presented here describe a somewhat modified "rapid" COD test, in which one analysis can be completed in less than 30 min at a chemical cost of approximately 25 percent of the Standard Methods test.

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This "rapid" COD test is not intended as a substitute for the Standard Methods COD procedure because in some cases it will not be as efficient in the oxidation of certain organic compounds. However, results of extensive studies conducted at several analytical laboratories indicate this method to be simple, rapid, and sufficiently reproducible for routine use in wastewater analysis. Therefore, the "rapid" method warrants serious consideration by treatment plant operators and laboratory personnel for most analytical applications.

Previous work

The "rapid" COD procedure, as adapted, is based on a modified COD test proposed by Pepinsky *et al.*[2] The original work of Pepinsky reported the use of iodide, starch, and sodium thiosulfate in the titration, whereas the proposed method employs ferrous ammonium sulfate and ferroin indicator. Other modifications of the original methodology include incorporation of sulfate salts of silver and mercury.

Silver sulfate was not used as a catalyst in most of the work reported by Pepinsky.[2] An interest in the effect of this catalyst on the oxidation of various substrates was the major reason for this investigation. Also, Dobbs and Williams' results[3] on the use of mercuric sulfate to eliminate the effects of chlorides have been incorporated into the test.

Proposed method

The following is a brief description of the proposed method. A detailed list of chemicals and laboratory procedure is given at the end of the paper.

TABLE 1—RAPID COD TEST RESULTS

Compound	COD (mg/L) Theoretical*	COD (mg/L) Rapid method	COD (mg/L) Std. method	Ratio (%) rapid method/ std. method	Std. deviation
Glucose	1299	1264	1258	100.4	5.6
Starch		1152	1111	103.8	3.3
Cellulose		1214	1206	100.6	107.0
Pyridine	2185	36	0		
Acetone		1430	1433	99.8	26.4
Ethanol	821	715	777	92.0	10.4
Lactic acid		960	1027	93.5	20.9
Acetic acid	772	729	727	100.3	9.0
Acetic acid	100	98	100	98.0	11.2
Oleic acid		745	731	102.0	3.3
Stearic acid		800	917	87.4	63.4
Glycine	1243	250	505	49.6	22.6
Phenylalanine	1390	1330	1347	98.9	19.8
Nutrient broth		492	536	91.8	17.2
Activated sludge		2150	2266	94.9	37.7
Digested sludge		1409	1441	97.7	8.9
Raw sewage		183	202	90.6	13.8
Settled sewage		172	183	94.0	14.2

* Computed using the weight of compound.

TABLE 2—EFFECT OF TEMPERATURE ON DICHROMATE

Temp. (°C)	Titration (ml)
30	24.76
165	24.61
170	24.63
180	24.42
190	24.02

TABLE 3—TEMPERATURE EFFECT ON GLYCINE OXIDATION

Temp. (°C)	COD (mg/L)	Ratio (%) rapid method/ std. method
165	295	47
180	465	75
200	499	80
Std. method	624	

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The "rapid" COD procedure involves the addition of the waste sample to a flask containing mercuric sulfate. After mixing, 25 ml of a dichromate-acid-silver sulfate mixture is added, mixed, and placed on a preheated hot plate. A thermometer is inserted in the flask, and the temperature is checked as the mixture is heated. When 165°C is reached, the sample is withdrawn. (Care must be employed in heating the sample because the temperature can rise very quickly.) Water is then added cautiously, and after cooling, the solution is titrated with ferrous ammonium sulfate in the presence of ferroin indicator.

Compounds and substrates

For the test to be general and widely applicable, selection was made of a variety of organic compounds that would be indicative of the typical constituents of domestic and industrial wastes. Included are representative compounds of the three major constituents of domestic sewage, the carbohydrates, fats, and proteins. Volatile compounds tested included ethanol and acetone; and among the difficult-to-degrade compounds were acetic acid, glycine, and pyridine. For representation of treatment plant wastewaters, raw sewage, settled sewage, activated sludge, and anaerobic digester effluent were also analyzed.

A series of runs was made using the selected organic compounds and substrates. Each run consisted of ten replicates of the substrate, five distilled water blanks, and two standards, all by the "rapid" COD method, as well as four analyses by the Standard Methods COD test.[1] The results, along with statistical analyses, are given in Table 1. The statistical data given in this table refer to the ten replicate samples that were performed using the "rapid" COD test.

Temperature effects

The temperature to which the sample and chemicals are heated is an important consideration. In the laboratory work described, particular care was taken so that the COD analyses were carried to 165±1°C. It has been found that an increase in the temperature results in the consumption of more potassium dichromate as well as an increase in the oxidation of the organic material present. Table 2 shows the effect of temperature on the destruction of dichromate. These results were obtained by substituting water for the sample in Step 2 of the detailed procedure. Five analyses were made at each temperature, and the average value is given in Table 1.

Not all compounds are oxidized with the same ease, hence, a series of runs using glycine were made to demonstrate the increased oxidation of hard-to-oxidize compounds with increasing temperatures. Four replicate samples at each of several temperatures were analyzed using the "rapid" method. The average results of these analyses compared with those obtained by the Standard Method COD test are shown in Table 3.

Discussion

With the exception of pyridine and glycine, all the substrates studied were oxidized to a satisfactory level when compared to the Standard Methods procedure. However, even the Standard Methods procedure was not very effective in oxidizing pyridine and glycine.

Analyzing the general classes of compounds, it is obvious that the carbohydrates are oxidized very efficiently as compared to the reaction in the Standard Methods test. Of the organic acids tested, all were degraded in excess of 90 percent, except for stearic acid, which was oxidized to 87 percent of the Standard Methods test. Proteinaceous compounds, including nutrient broth, the amino acid phenylalanine, and cellular material of activated and digested sludges, were all oxidized to over 90 percent of the Standard Methods results. Only the amino acid glycine was oxidized to a much lower level.

Cellulose and stearic acid had standard deviations of 107 and 63, respectively, and represent the largest values encountered of all the compounds tested. It is believed, however, that this large deviation is caused primarily by the difficulty of measuring precisely the small amounts of these materials used in the test rather than in the chemical oxidation.

Some concern had been expressed concerning the possible loss of volatile organics from the sample because heating is accomplished in an open flask without use of a reflux condenser. The low standard deviations for almost all of the work indicates quite conclusively, however, that the volatility of the organics tested, including compounds such as acetone and ethanol, is not a serious source of error.

Results of the "rapid" method at COD concentrations of less than 100 mg per L tend to vary in duplicate samples. This is indicative even at the 100 mg per L concentration of acetic acid, where the standard deviation was ten percent of the mean value. The variation, however, can be reduced by using larger samples, such as 10, 20, or more ml. Longer heating times are then required because sufficient water must first evaporate before the temperature of the solution can reach 165°C. Further work in this direction, along with high temperature effects, is being continued.

The procedure has great potential in treatment plants or in cases where routine control is required. For any particular type of analysis, for example an effluent from a treatment unit, a factor between the "rapid" method and Standard Method may be determined by running 10 samples with each method. This factor can then be applied on all future samples run by the "rapid" method. As an occasional check, several samples should be run by Standard Methods to check any change in the factor resulting from minor alterations of the procedure or change in the wastewater characteristics.

The rapidity of the method is the major advantage of the procedure. Generally, a trained laboratory technician can heat 20 samples in less than 30 min, as several samples can be placed on the hotplate at one time. Another advantage in the method lies in the fact that the number of samples run is not limited by the number of condensers available, as in the Standard Methods procedure. The rapid method is less costly and requires the use of only small volume samples. The disadvantages of the rapid method are that a standard must be run, temperature control is required, and reliability is poor in dilute samples.

There are many other variables that should be studied in the procedure but there is little doubt that the proposed "rapid" method is excellent for routine COD analyses. More than 90 percent oxidation can be obtained for most substrates.

Chemical reagents

1. Dichromate-acid solution 0.05*N*. Dissolve 5 g $K_2Cr_2O_7$ and 20 g $AgSO_4$ in a solution consisting of 1 L each of concentrated H_2SO_4 and H_3PO_4.
2. Ferrous ammonium sulfate, 0.05*N*. Dissolve 20 g of $Fe(NH_4)_2\ (SO_4)_2 \cdot 6H_2O$ in distilled water, add 20 ml concentrated H_2SO_4 and dilute to 1 L. This solution is standardized against 0.050*N* $K_2Cr_2O_7$ prior to use.
3. Ferroin indicator solution (as in Standard Methods)
4. Mercuric sulfate
5. Dichromate standard, 0.05*N*. Dissolve 2.4518 g $K_2Cr_2O_7$ dried, primary standard grade, in distilled water and dilute to 1.00 L.

All of the above are ACS reagent grade chemicals unless otherwise noted.

Procedure

1. Add approximately 0.3g of $HgSO_4$ to a 500 ml Erlenmeyer flask. (With a little practice, this may be measured directly from the tip of a spatula without weighing.)

2. Pipet 5.0 ml or a suitable aliquot of the sample diluted to 5 ml into the flask. (Approximately 2 to 8 mg COD in sample—400 to 1600 mg per L—for best results.) Mix well.

3. Two blanks are run by substituting 5.0 ml distilled water for the sample and continuing with steps 4 through 8. The average of the two titrations is used in the calculations.

4. Carefully add 25.0 ml of the dichromate-acid solution to the flask and swirl the contents. (An automatic dispenser is recommended.)

5. Handling the flask by the neck, place the Erlenmeyer flask on a preheated hot plate and heat to 165± 1°C. A thermometer should be immersed in the solution, and frequent swirling should be employed. The temperature is read by tilting the flask so that the thermometer is sufficiently immersed. Approximately 5 min is required to reach 165° C, at which time the flask is removed from the hot plate. With experience, from 4 to 5 flasks may be heated at one time.

6. Cautiously add approximately 300 ml of distilled water. (The addition of water to the very hot acid mixture can induce vigorous boiling and spattering.)

7. Cool the solution in a water bath to ambient temperature.

8. Add 5 drops of the ferroin indicator and titrate to an orange end point with ferrous ammonium sulfate.

9. Two standards are run by adding 25 ml of 0.050*N* dichromate, acidifying with 20 ml concentrated sulfuric acid, and continuing with steps 6, 7, and 8.

Calculations

The COD is calculated as follows:

$$N = \frac{(25)(0.05)}{\text{ml from step 9}} \qquad \text{Eq. 1}$$

where N = normality of $Fe(NH_4)_2\ (SO_4)_2$

COD (mg/L) =

$$\frac{N \times 8000 \times (\text{ml from step 3} - \text{ml from step 8})}{\text{ml sample}} \qquad \text{Eq. 2}$$

REFERENCES

1. "Standard Methods for the Examination of Water and Wastewater," 12th ed., New York, American Public Health Assn., Inc., 1965.
2. Pepinsky, . B., Porges, N., and Hoover, S. R., "Application of a Rapid Chemical Oxygen Demand Test for Determining Organic Pollutions," Proc. 6th Industrial Waste Conference, Purdue Univ., Series 76, 1951, pp. 367-374.
3. Dobbs, R. A. and Williams, R. T., *Anal. Chem.* **35:** 1064 (1963).

KEY WORDS

Laboratory Methods and Control.

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APPENDIX II

COMPARISON OF VARIOUS NITROGEN SOURCES IN ANAEROBIC TREATMENT

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COMPARISON OF VARIOUS NITROGEN SOURCES IN ANAEROBIC TREATMENT

Robert Gasser and John S. Jeris

Anaerobic treatment is a process which has been utilized primarily in domestic waste treatment to stabilize the concentrated sludges withdrawn from primary and secondary sedimentation tanks. In recent years, this process has been employed increasingly for the treatment of industrial wastewaters. Unlike domestic wastewater, industrial wastes frequently lack the nutrients essential for the proper operation of biological treatment systems. In such cases, auxiliary nutrient supplementation must be incorporated in the design. This investigation concerns the treatment of an industrial waste which was deficient in nitrogen.

Nitrogen is essential for the proper growth of the organisms involved in the biological treatment of organic wastes. Sanders and Bloodgood (1) reported that the nitrogen-to-carbon ratio in the substrate fed to an anaerobic digester should be in the order of 0.06 and that a value below this level would cause digester failure. Speece and McCarty (2) reported that the nitrogen requirement is dependent on the substrate and the sludge retention time. They indicated that a carbohydrate substrate requires more nitrogen than proteinaceous or fatty substrates due to the greater availability of free energy.

While it is important to know the amount of supplemental nitrogen required, it is of equal importance to use the proper form of nitrogen. Brezonik and Lee (3) studied the effects of nitrogen nutrients in the form of NH_4Cl, $NaNO_3$, and NH_4NO_3 and concluded that ammonia nitrogen was assimilated most readily into cell mass, while nitrate sources were converted extensively to nitrogen gas and thus not totally available for cell synthesis. They also reported that relatively low concentrations of nitrate caused bacteriostasis.

Most of the previous work that has been reported regarding the effects produced by nitrogen nutrients utilized biological systems that were operating efficiently or used high concentrations of nitrogen. The objective of this investigation was to determine the effects produced in a nitrogen limiting system using various concentrations of different nitrogen sources.

Operation of Biological Systems

Initially, this study was undertaken for a private industrial concern. The purpose was to compare the effects of various nitrogen compounds on laboratory scale anaerobic lagoons being fed a waste deficient in nitrogen. This type of treatment was simulated using a ten-liter vessel filled to the six-liter mark (Figure 1). Seven units of this type initially were seeded with sludge from a municipal digester in a ratio of one-third sludge and two-thirds warm tap water. The units were operated for an extended period of time prior to the compilation of data in order to acclimate the sludge to the waste. To assure that most of the initial seed material was purged from

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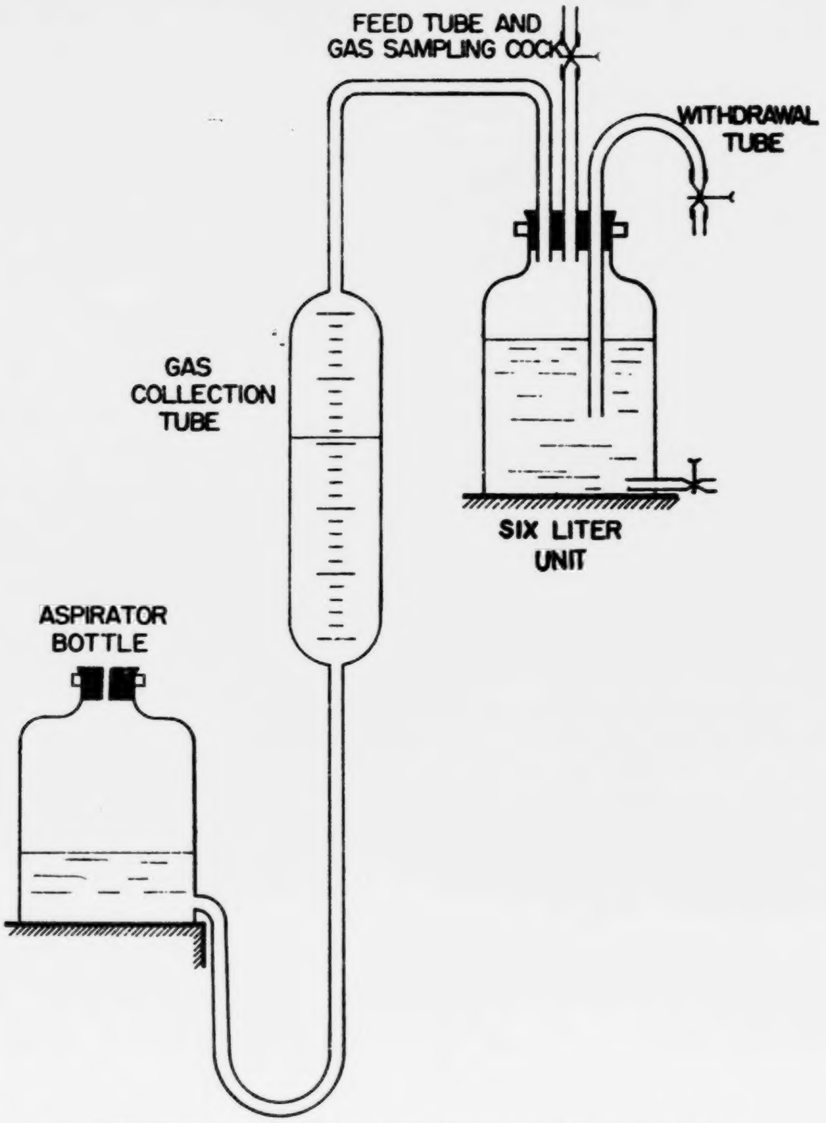

FIGURE 1.—The batch-fed units had ten-liter capacities.

the systems, sludge samples were withdrawn from the bottom of the units for a period of one month after initial start-up. The units were maintained in a walk-in type incubator at a temperature of 35°C±0.5°C. After a daily withdrawal of sludge sample from the top portion of the vessel, feed was added. The only mixing that occurred was caused by dispersion of the feed substrate as it entered the vessel. Following this phase of the study, tests were run on a mixed system resembling a batch-fed anaerobic digester. Prior to feeding substrate, the contents of the vessel were well mixed and a sample of sludge was withdrawn. After feeding, the contents again were well mixed to assure intimate contact of the substrate with the biological solids present. For simplicity, these systems will be referred to as "mixed" and the simulated lagoons as "unmixed." In both phases of this study the units had a 12-day hydraulic detention time.

Substrate and Additives

The substrate used in this study was composed primarily of cornstarch and corn syrup and was prepared in the laboratory to simulate the waste of the industrial plant. The waste was composed of a mixture having a COD of 7,400 mg/l which was 89-percent cornstarch and 11-percent corn syrup. Sufficient sodium bicarbonate and potassium hydroxide were added to maintain the pH in the neutral zone. Phosphorous was added along with other trace elements to assure proper growth of organisms. The only variables under consideration in this study were the nitrogen source and concentration.

Based on the work of Speece and McCarty (2), the nitrogen requirement for a carbohydrate feed with a sludge retention time of 12 days is approximately 20 to 25 mg of nitrogen for every gram of COD utilized. Using this data initially as a guideline and conservatively assuming 100-percent utilization of the substrate, half of the units were fed 20.8 mg N/g COD fed and the other half one-fifth of this concentration. Table I lists the concentrations and nitrogen sources fed to the "mixed" and "unmixed" systems.

Laboratory Analyses

In order to control and measure the performance of the anaerobic units the following analyses were performed weekly:

(*a*) pH
(*b*) Alkalinity
(*c*) Volatile acids
(*d*) Soluble COD (effluent and substrate)
(*e*) Total COD (effluent and substrate)
(*f*) Organic nitrogen
(*g*) Ammonia nitrogen
(*h*) Gas composition

TABLE I.—Daily Nitrogen Additions

Unit No.	Nitrogen Source	mg N/g COD fed	
		Unmixed	Mixed
1	Control	0	0
	NH_4Cl	20.8	20.8
3	NH_4Cl	4.2	8.65
4	$NaNO_3$	20.8	20.8
5	$NaNO_3$	4.2	8.65
6	NH_4NO_3	20.8	20.8
7	NH_4NO_3	4.2	14.6

All analyses were performed in conformance with "Standard Methods for the Examination of Water and Wastewater" (4) with the exception of analyses (*d*), (*e*), and (*h*). The chemical oxygen demand determinations were made following a rapid method (5), the results of which compare favorably with the method outlined in "Standard Methods." Gas analysis was performed using a Wilkins Aerograph Gas Chromatograph Model A-90P.*

Nitrogen Balance

Since the concentration of nitrogen was the controlling factor in this study, it was desirable to make a material balance on the influent and effluent nitrogen concentrations. The amount of nitrogen added in the influent of each unit was a known amount (Table I). Analyses were performed routinely on the effluent for ammonia, organic nitrogen, and nitrogen gas. Theoretically, the sum of the nitrogen in the effluent should equal the concentration fed. However, nitrate and nitrite were not detected during the study. In the unmixed phase it was difficult to make a reasonable balance since the system was not stable. Gas bubbles rising from the bottom disturbed the settled sludge and carried particulate matter into the supernatant liquor. There was a continual buildup of nitrogen in the bottom sediment as the nitrogen was converted to settling cell matter. As the cells died and lysed, release of nitrogen occurred for reuse.

A nitrogen balance was made on the mixed systems (Table II). The control unit and the units which were fed ammonia (units 1, 2, and 3) should have no nitrogen in the gaseous fraction. However, they had an aver-

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* Varian Aerograph, Walnut Creek, Calif.

TABLE II.—Nitrogen Balance—Mixed Phase

Unit No.	Nitrogen Source	Nitrogen Fed (mg/l)	NH_3-N (mg/l)	Org-N (mg/l)	N_2-N (mg/l)	Recovery (%)
1	Control	2.8	0	9.8	—	350
2	NH_4Cl	156.8	38.8	106	—	92
3	NH_4Cl	66.8	0	57	—	85
4	$NaNO_3$	156.8	0	66	80.5	94
5	$NaNO_3$	66.8	0	57	28.6	128
6	NH_4NO_3	156.8	0	104	56.6	103
7	NH_4NO_3	110	0	65	21.2	78

age of about one percent nitrogen in the effluent gas. This concentration of nitrogen gas can be attributed to leaks, air passing through rubber tubing, and feeding or gas sampling techniques. Therefore, a correction of one-percent nitrogen gas was made on the gas readings of all units fed nitrates.

Greater than 90-percent recovery was achieved for the units which were fed the high concentration of nitrogen, while the balance was not as good for the other units. A possible explanation for the poorer recovery in the units fed lesser amounts of nitrogen is that the correction factor needed to account for the leakage of air was not one percent. Since the amount of nitrogen fed to these units was smaller, a small error in the correction factor is significant.

Results

Control Unit

Unit one, the control unit, received all the nutrients necessary to support microbial activity with the exception of nitrogen. Chemical analyses of the feed substrate showed that there was a small amount of nitrogen (2.8 mg/l) being fed daily. Aside from this minimal concentration, there was no supplemental nitrogen fed to this unit. While operating as a lagoon (unmixed), the average daily gas produced by this unit was 1,050 ml/day. Although the control was the least efficient of all the units, it did achieve 54.5-percent removal of the influent COD. When the operation was changed and mixing incorporated, the gas production of the control decreased to 690 ml/day and the efficiency of removal dropped to 32.4 percent. The effluent sludge contained no ammonia, and the organic nitrogen content, which is an indication of the biological mass present, reached the lowest level encountered in the study.

Results of Unmixed Systems

Table III presents the averages of data collected during the unmixed phase of this study. The units which received the highest concentration of nitrogen had the lowest volatile acid concentrations, the highest concentrations of organic nitrogen, and the

TABLE III.—Equilibrium Data—Unmixed Phase

Unit No.	Nitrogen Nutrient	Nitrogen Fed (mg/l)	Effl. NH_3-N (mg/l)	Effl. Org-N (mg/l)	Effl. N_2 Gas (%)	Volatile Acids (mg/l)	COD Removed (%)
1	Control	2.8	0	4.6	1.0	2,410	54
2	NH_4Cl	156.8	35	91	0.5	425	80
3	NH_4Cl	33.6	0	34	1.5	1,240	68
4	$NaNO_3$	156.8	0	46	3.9	760	78
5	$NaNO_3$	33.6	0	13	2.5	2,180	67
6	NH_4NO_3	156.8	12	92	2.3	235	82
7	NH_4NO_3	33.6	0	33	1.7	1,260	66

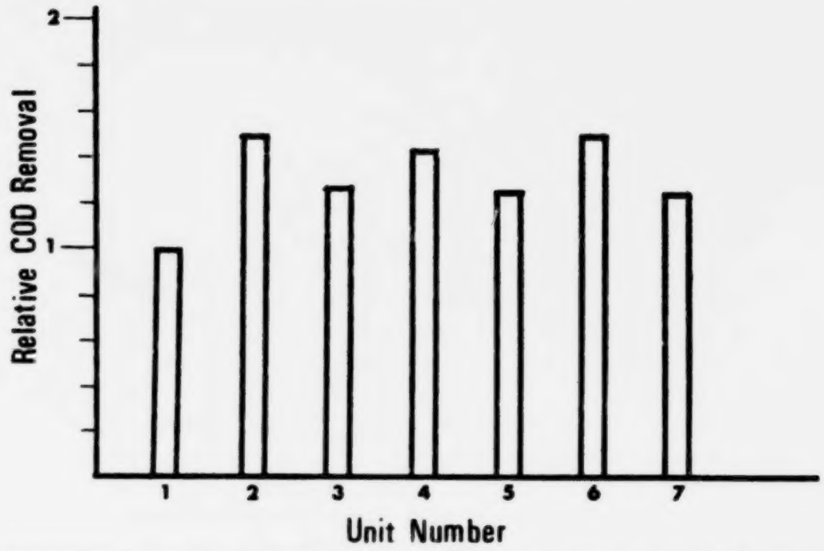

FIGURE 2.—Relative COD removal efficiencies for the unmixed phase were 20 to 55 percent higher than the control value (Table I).

highest efficiencies. The units which received nitrate as a nutrient source had higher concentrations of nitrogen in the effluent gas produced. Excess nitrogen, as measured by unassimilated ammonia in the effluent, was present in units 2 and 6. A COD value of unity was assigned arbitrarily to the control unit. All of the nitrogen-fed units had efficiencies of 20 to 55 percent higher (Figure 2) than the control unit, which received no nitrogen additive. The 3 units which received 20.8 mg N/g COD fed, operated with about 80-percent efficiency, while the 3 units which received one-fifth as much nitrogen operated at approximately 67-percent efficiency.

During this unmixed phase, bottom samples were withdrawn from each unit and analyses performed to determine the ammonia, organic nitrogen, volatile solids, and efficiency of COD removal. These data (Table IV) indicate that the units which had the highest ratio of organic nitrogen to total volatile solids also were the most efficient as measured by COD removal.

Results of Mixed Systems

The major part of the study was concerned with anaerobic treatment in the mixed digesters (Table V). Since the units originally were operated in a quiescent state, there was a build up of cells in the bottom sludge which was indicated by the organic nitrogen content of the effluent liquor and of the bottom sludge. When mixing was initiated, the sludge concentration be-

TABLE IV.—Analysis of Bottom Sludge—Unmixed Phase

Unit No.	Nitrogen Nutrient	Nitrogen Fed (mg/l)	NH_3-N (mg/l)	Org-N (mg/l)	mg Org-N/g VS	COD Removed (%)
1	Control	2.8	0	60	14	54
2	NH_4Cl	156.8	37	340	87	80
3	NH_4Cl	33.6	0	270	56	68
4	$NaNO_3$	156.8	0	585	74	78
5	$NaNO_3$	33.6	0	210	44	67
6	NH_4NO_3	156.8	20	335	93	83
7	NH_4NO_3	33.6	0	240	40	68

TABLE V.—Equilibrium Data—Mixed Phase

Unit No.	Nitrogen Nutrient	Nitrogen Fed (mg/l)	Effl. NH_3-N (mg/l)	Effl. Org-N (mg/l)	Effl. N_2-N (%)	Volatile Acids (mg/l)	COD Removed (%)
1	Control	2.8	0	9.8	0.8	3,200	32
2	NH_4Cl	156.8	39	106	0.5	350	74
3	NH_4Cl	66.8	0	57	1.4	1,030	71
4	$NaNO_3$	156.8	0	66	5.5	2,210	43
5	$NaNO_3$	66.8	0	57	3.8	3,450	19
6	NH_4NO_3	156.8	0	104	3.0	1,400	60
7	NH_4NO_3	110	0	65	3.0	3,730	21

came uniform throughout the units and, eventually, the excess sludge that had built up previously was washed out. The units were operated until there was a constant amount of organic nitrogen in the effluent from each digester. Equilibrium conditions were approached during the 17th to 21st weeks following the initiation of mixing (Figure 3). Unit 2, which had not reached equilibrium prior to being broken accidentally, also was assumed to be approaching equilibrium.

As in the unmixed systems, the mixed digesters which received the highest concentrations of nitrogen had the lowest volatile acid concentrations, the highest concentrations of organic nitrogen, and the highest efficiencies. However, unlike the unmixed study, there was a marked difference in the efficiency of COD removal, dependent on the type of nitrogen added (Figure 4). Five of the nitrogen-supplemented digesters performed more efficiently than the control. Of the units which received 20.8 mg N/g COD fed, the NH_4Cl digester was 2.27 times as efficient, the NH_4NO_3 unit was 1.85 times as efficient, and the $NaNO_3$ unit was 1.31 times as efficient as the control unit.

Significantly, the NH_4Cl unit which received only 8.65 mg N/g COD fed was 2.20 times as effective as the control. The remaining two nitrogen-supplemented units performed less efficiently than the control unit. The units which received $NaNO_3$ as a nitrogen source performed quite poorly. About 50 percent of the nitrogen which was fed to these units was converted to nitrogen gas and thereby rendered unavailable for cell growth (Table V). Similarly, relatively large concentrations of nitrogen were present in the

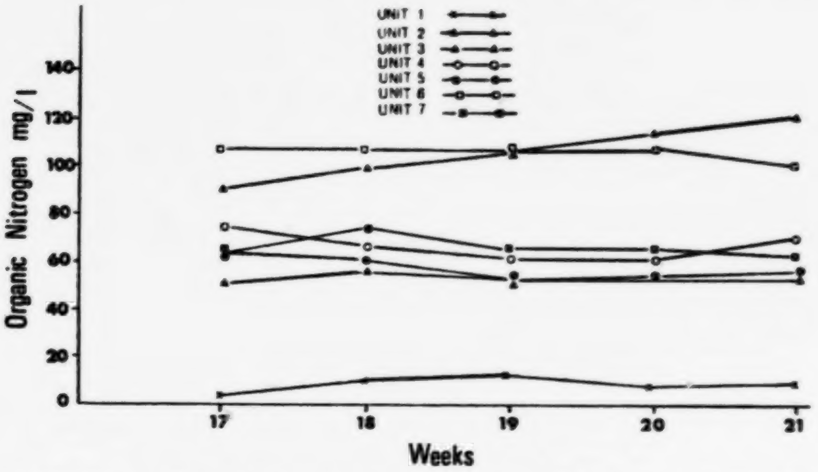

FIGURE 3.—Organic nitrogen equilibrium conditions were approached during the 17th to the 21st weeks (Table II).

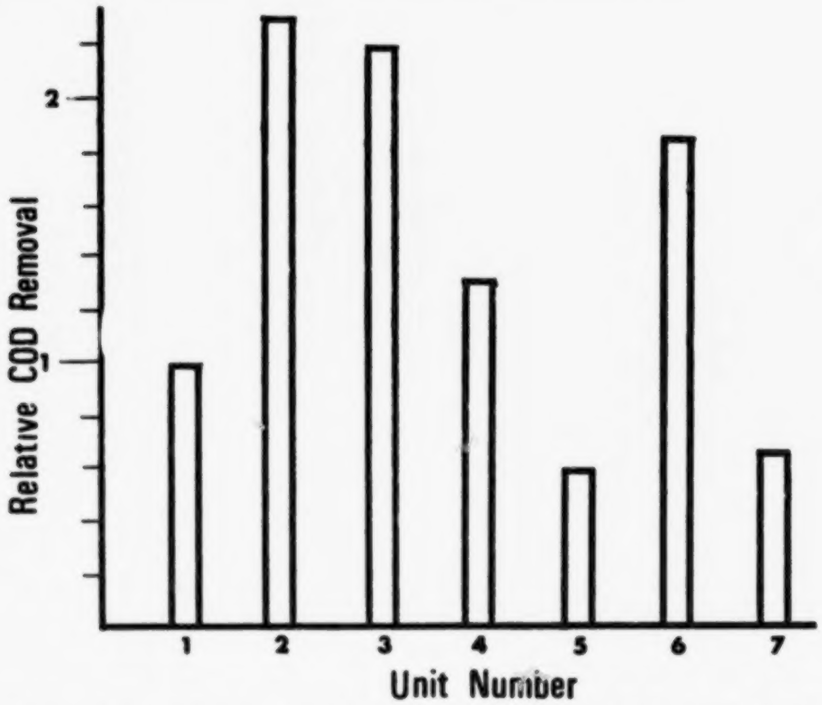

FIGURE 4.—Relative COD removal efficiencies for the mixed phase during the 17th to the 21st weeks (Table II).

effluent gas of the NH_4NO_3 fed digesters.

A comparison was made in this phase of the study between the soluble effluent COD and the volatile acid concentration expressed in terms of its COD (Table VI). The COD of the volatile acids was computed based on acetic acid. In most cases the ratio of volatile acids to soluble COD was about 0.85, indicating that almost all of the substrate had been converted to volatile acids or other non-soluble matter. However, for complete stabilization under anaerobic treatment, volatile acids must be converted to the end products—methane and carbon dioxide. By comparing the quantity of methane gas generated in the nitrogen-fed units, only the ammonium chloride-fed units were effectively converting the volatile acids to methane and carbon dioxide. As the majority of the soluble COD remaining is due to volatile acids, it appears that the methane-forming organisms cannot compete as effectively as the other organisms present for the available nitrogen.

TABLE VI.—Volatile Acids and Soluble COD—Mixed Phase

Unit No.	Volatile Acids (mg/l)	Soluble COD (mg/l)	$\frac{\text{VA COD}}{\text{Sol. COD}}$	Daily CH_4 Produced (ml)
1	3,200	4,830	.71	180
2	350	505	.74	738
3	1,030	1,275	.86	713
4	2,210	2,915	.81	276
5	3,450	3,530	1.04	65
6	1,400	1,750	.85	520
7	3,730	4,715	.85	63

Nitrogen Requirements

The purpose of this study was to determine the most effective nitrogen source and the concentration necessary for the treatment of an industrial waste. For the nitrogen-COD relationship derived, it is assumed that the quantity of nitrogen used is equal to the organic nitrogen (Table VII). The units which received the higher concentration of nitrogen used approximately 21

TABLE VII.—Nitrogen Requirement

Unit No.	Nitrogen Fed (mg/l)	Org-N (mg/l)	COD Removal	mg N used/g COD used	mg N Fed/g COD used
1	2.8	9.8	32	4.1	2.3
2	156.8	105.8	74	19.4	28.3
3	66.8	56.6	71	10.8	12.3
4	156.8	66.4	43	21.2	50.0
5	66.8	57.2	19	36.5	45.2
6	156.8	104.0	60	23.4	34.7
7	110	65.4	21	48.3	69.0

mg N for each gram of COD removed. This value is in agreement with that obtained by Speece and McCarty (2). Unit 3, the unit which received a lower concentration of ammonium chloride, required only 10.8 mg N for each gram of COD used to accomplish satisfactory COD removal. This is significant since it has been reported previously that almost twice this concentration is required to support microbial activity. The high nitrogen-used/COD-removed ratios obtained for units 5 and 7 are most likely an indication of the synthesis of organisms deriving their energy requirements from denitrification and starch breakdown to volatile acids. The efficiency of COD removal of these units was approximately 20 percent. The differences between the nitrogen used and nitrogen fed per gram of COD utilized is due to the nitrogen loss as a gas, ammonia in the effluent, and differences in the material balance. Although the control shows the least nitrogen required for each gram of COD used, the low efficiency negates its significance.

Discussion

In this study the control unit was operated with a minimal amount of nitrogen (2.8 mg/l) in the substrate. However, chemical analyses indicate that 9.8 mg/l nitrogen was present in the effluent liquor in the form of organic nitrogen. Although this is still a small concentration, the result was unexpected. One possible explanation for this occurrence is that under the extreme nitrogen limiting conditions, nitrogen fixing bacteria converted nitrogen gas into cell nitrogen.

The results of the anaerobic lagoon (or unmixed phase of this study) indicate that any of the three sources of nitrogen investigated will fulfill adequately the nutritional nitrogen requirements. Since the units were not mixed, solids separation occurred. As nitrogen is converted into cell mass, it is incorporated into the settleable sludge. The high concentration of bottom solids indicates a high solids and nitrogen retention time, with the availability of a larger number of organisms to utilize the nutrients and substrate. Under these conditions, less nitrogen is required. In addition, as the nitrogen is released through cell lysis, it can be reused. Under the conditions and concentrations studied, the efficiency of COD removal was more dependent on retaining the nitrogen in the biological units than on the nitrogen source. Under more severe limiting nitrogen conditions, the ammonia fed units probably would perform more satisfactorily, since there is no loss of nitrogen through the formation of gaseous nitrogen.

In the mixed phase of the study, the digesters receiving ammonia as a nitrogen source performed more efficiently than any of the other units. This was expected since ammonia nitrogen is at its most reduced form and, therefore, more readily available for cell synthesis. The units fed NH_4NO_3 and $NaNO_3$ did not perform as efficiently since the nitrate must be reduced to the ammonia state before it can be utilized for synthesis. In the process of reducing nitrate to ammonia, a great deal of nitrogen is wasted by conversion to nitrogen gas. In the four units which received nitrate as a nitrogen source, 30 to 50 percent of the nitrate fed was converted to gaseous nitrogen.

Significantly, it was noted in both the mixed and unmixed systems that the volatile acid concentration was higher in the units receiving the lower amounts of nitrogen. Since the majority of the soluble COD remaining in these unit was in the form of volatile acids, it may be concluded that the "first stage" organisms, as often considered in anaerobic treatment, were converting the carbohydrate substrate to volatile acids. As high concentrations of volatile acids remained in the nitrogen limiting units, it appears that the so-called "first stage" organisms compete more effectively for the nitrogen that is available. If nitrogen is limiting to the methane formers, the ultimate breakdown of the volatile acids to the end products—methane and carbon dioxide—is greatly reduced, as confirmed in the experimental work.

In biological systems maintained on non-proteinaceous substrates, the relative mass of the organisms present may be indicated by the organic nitrogen concentration. The higher the concentration of organic nitrogen, the greater the mass of cells. In the mixed phase of the study, the organic nitrogen present in the ammonium chloride-fed units was in the ratio of one-to-two, although the efficiency did not vary significantly. It is conceivable that since one unit received an excess amount of nitrogen, some of the nitrogen was assimilated by the microbes and stored as cell mass and actually could have survived with lesser nitrogen. This also was true in the unmixed phase of the study. Conversely, unit 6, the ammonium nitrate unit which had a high concentration of organic nitrogen, performed poorly compared with the ammonia fed units. In this case nitrogen probably was being utilized by denitrifying organisms responsible for the conversion of nitrate to ammonia.

Figure 5 shows that units 5 and 7 performed more poorly than the control. The reason for this is not known. However, it has been demonstrated that nitrate, at fairly low concentrations, impedes the performance of the methane formers (3). It also is conceivable that nitrate or other metabolic end products retarded the low methane population that could be supported under the nitrogen limiting conditions imposed. Further, the bacterial populations and species would be different for the nitrate and non-nitrate fed units. Nitrate, a highly oxidized compound, would allow denitrifiers and perhaps other organisms to grow.

The results of this study tend to indicate that anaerobic lagoons (unmixed) are more efficient in removing COD than digesters (mixed). This is somewhat misleading since it has been proven that mixing improves the operation of the anaerobic treatment process. It is difficult, however, to set up in a laboratory a unit which is exactly similar to the prototype. For example, in the "mixed" phase of the study the units were not mixed continually, but rather, mixed manually prior to and following the addition of substrate. Similarly, in the "unmixed" or lagoon type study the influent was well dispersed over the surface of the vessel. In an actual lagoon the influent normally is introduced into a relatively small area. Also there was a significant difference in the quality of the effluent from each type of unit—the effluent from the digesters contained a mixed sample with settleable solids present, whereas the effluent from the lagoons was taken from the supernatant and therefore contained fewer solids. Keeping this in mind, it is not possible to make a comparison between the results of the two types of operation. Rather, the results of these phases of study should be analyzed separately.

Conclusions

1. The source of nitrogen—ammonia, nitrate, or ammonium nitrate—has no significance in the operation of the unmixed (lagoon) system.

2. Less nitrogen is required in the unmixed (lagoon) system than in the mixed (digester) systems to obtain satisfactory COD removal.

3. Nitrogen in the ammonia form is superior to nitrate under nitrogen limiting conditions in the mixed (digester) system.

4. Almost half the nitrogen fed to the mixed systems as nitrate nitrogen is wasted by conversion to gaseous nitrogen.

5. Methane formers do not compete favorably with other anaerobic and facultative organisms for nitrogen.

6. Using nitrogen limiting conditions, less nitrogen is required for satisfactory operation under mixed and unmixed conditions than has been reported previously.

Acknowledgments

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References

1. Sanders, F. A., and Bloodgood, D. E., "The Effect of Nitrogen-to-Carbon Ratio on Anaerobic Decomposition." This Journal, **37**, 12, 1741 (Dec. 1965).
2. Speece, R. E., and McCarty, P. L., "Nutrient Requirements and Biological Solids Accumulation in Anaerobic Digestion." In "Advances in Water Pollution Research." Proc. 1st Intl. Conf. Water Poll. Res., Pergamon Press, Ltd., London, England, Vol. 2, 61 (1964).
3. Brezonik, P. L., and Lee, G. F., "Sources of Elemental Nitrogen in Fermentation Gases." *Intl. Jour. Air and Water Poll.* (Brit.), **10**, 145 (1966).
4. "Standard Methods for the Examination of Water and Wastewater." 12th Ed., Amer. Pub. Health Assn., New York (1965).
5. Jeris, J. S., "A Rapid COD Test." *Water and Wastes Eng.*, **4**, 89 (1967).

APPENDIX III

GLUCOSE DISAPPEARANCE IN BIOLOGICAL TREATMENT SYSTEMS

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Glucose Disappearance in Biological Treatment Systems

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Abstract

Jeris, John S. (Manhattan College, Bronx, N.Y.), **and Raul R. Cardenas, Jr.** Glucose disappearance in biological treatment systems. Appl. Microbiol. **14:**857–864. 1966.—Laboratory scale anaerobic and aerobic treatment units were conditioned with a daily slug-feed of glucose. After a period of acclimation and stabilization, glucose disappearance was monitored continuously after the slug feed. A continuous sampling apparatus is described. Mathematical analysis of the data indicate zero-order reactions for both biological treatment systems.

Continuous studies on pure substrates are not always easy to perform. Many such studies, in fact, must rely on closely spaced intermittent samplings or an indirect analysis for the desired substrate. Inasmuch as the sanitary engineer has a great interest in the rate of substrate uptake (or its disappearance) in biological systems, intermittent sampling does not always provide the investigator with sufficient information to develop reliable engineering design data. When applicable, continuous monitoring for a known specific substrate by enzymatic or chemical analysis provides a valuable tool in investigating the kinetics of substrate removal.

In this paper we describe a study in which anaerobic and aerobic treatment systems, previously conditioned to glucose as a substrate, were continuously monitored for glucose concentration by enzymatic analyses.

Materials and Methods

Autoanalyzer. The instrumental system, as well as the manifold used for the automated analysis of glucose, was the Autoanalyzer (Technicon Instruments Corp., Chauncey, N.Y.).

Automated glucose method. Methodology for glucose analysis was essentially that of Hill and Kessler (3). A flow diagram is presented as Fig. 1. For the anaerobic system, samples were withdrawn continuously from a moving sample stream taken from the anaerobic digester. In the aerobic system, continuous samples were withdrawn directly from the aeration compartment of the treatment unit. In either case, the samples were then filtered and diluted with an air-segmented solution of 1.61% sodium sulfate. After mixing, this solution was dialyzed against an air-segmented solution containing Triton X-100 (isooctyl phenyl ether of polyethylene glycol; Rohm & Haas Co., Philadelphia, Pa.). The dialysate was then combined with the enzyme-dye mixture and was incubated at 37 C. Absorbancy was measured at 460 mμ in a 15-mm tubular flow cell. The reagents used were as follows.

Sodium sulfate solution was anhydrous Na_2SO_4, 16.1 g per liter. Sodium sulfate-Triton X-100 solution was anhydrous Na_2SO_4, 16.1 g, plus 5 ml of Triton X-100 per liter. The enzyme preparation consisted of 25 g (1,500 units per g) of glucose-oxidase (Dee-O Miles Chemical Co., Clifton, N.J.) and 200 mg of horseradish peroxidase (2,000 units per μg, or RZ unit of 1 or more; Worthington Biochemical Corp., Freehold, N.J.) mixed with 1 liter of 0.1 M phosphate buffer at *p*H 7.0 and filtered through Whatman no. 1 filter paper. For storage, the solution was filtered through a sterile membrane (Millipore Filter Corp., Bedford, Mass.) and was refrigerated aseptically in an amber bottle. The enzyme preparation may be kept in this manner without appreciable loss of activity for several weeks. The dye was prepared by dissolving 500 mg of *o*-dianisidine (3,3′dimethoxybenzidine, Eastman Organic Chemicals, Rochester, N.Y.) in 100 ml of ethyl alcohol. A 250-ml amount of glycerol was added, and the mixture was diluted to 1 liter with distilled water. The solution was filtered and stored in an amber bottle. Refiltration through Whatman no. 1 paper may be necessary on storage.

Glucose standards of 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 mg/liter of glucose were used to prepare the calibration curve. Two sets of standards were used for all runs, one immediately prior to the slug feeding of glucose, the other immediately after the uptake study. An average of the two standards was plotted on graph paper and the

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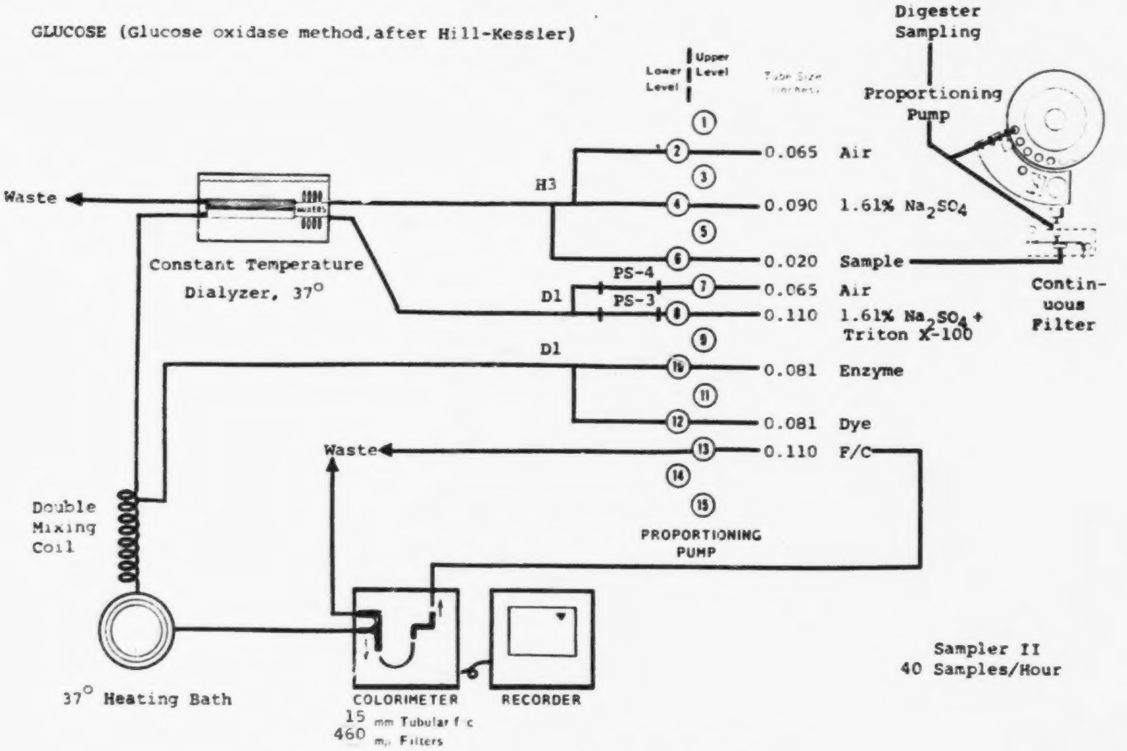

FIG. 1. *Autoanalyzer, flow diagram, glucose-oxidase.*

points were connected for the different glucose concentrations; a calibration curve was then constructed.

Anaerobic digester. The laboratory scale digester used is shown in Fig. 2, together with the continuous glucose-sampling equipment.

A 7.0-liter capacity laboratory scale unit was used for this study. Initially, the digester was seeded with 3 liters of screened primary digested sludge taken from a primary sewage-treatment plant. This sludge was mixed with 4.0 liters of tap water and was maintained at 35 C. The unit was fed nutrients daily until gas production and other parameters were observed to be constant. Detention time was maintained at 15 days by the daily liquid withdrawals. A diaphragm pump, connected as shown, maintained the unit uniformly mixed by continuous gas recirculation. Samples taken for intermittent analysis were withdrawn from the sample ports shown on Fig. 2 either directly from the tubing leading to the continuous-flow sample point or from the sample port near the bottom of the digester.

Prior to enzymatic glucose determination, the anaerobic digester was operated continuously over a period of 65 days. This enabled the system to reach equilibrium conditions. Process control tests included pH, alkalinity, volatile acids, and gas production.

Activated sludge unit. A Ludzack-type (9) activated sludge unit (Fig. 3) was employed for aerobic studies. This is a 5.5-liter unit containing a settling compartment and a porous stone diffuser. The unit was initially started with seed (2,000 mg/liter) from a municipal activated sludge-treatment plant. After about 1 week of operation under the conditions used, solids stabilized at approximately 1,700 mg/liter. Glucose dosage was maintained at 0.5 g chemical oxygen demand (COD) per day per g of Mixed Liquor Suspended Solids. Daily operation consisted of wasting 1,000 ml, followed by feeding of glucose, nitrogen, and phosphate buffer. Conditioning extended over a period of 21 days prior to the experiment. The pH was held in the range of 6.5 to 8.0.

Continuous sampling equipment. Sampling equipment from the digester to the Autoanalyzer is shown in Fig. 2. Continuous circulation for sampling of the anaerobic digester contents was accomplished by use of a Sigmamotor pump. Samples for glucose analysis were withdrawn continuously with a separate Autoanalyzer pump connected directly to the digester sample stream by a glass "T" with a ⅟₁₆-inch (0.16 cm; inner diameter) glass sampling tube inserted as shown. The tube at the junction of the T was adjusted to feed from the center of the moving digester stream. Since the sample stream contained particulate matter, solid material was filtered out through a continuous-flow filter by use of S & S no. 2043-A filter paper. However, further experiments have indicated filtering to be unnecessary.

The time required to move a sample from the center of the digester to the Autoanalyzer manifold assembly was approximately 3 min. The time required for sample recirculation from the digester past the continuous flow sample point and back to the digester was approximately 2.5 min.

Sampling from the activated sludge unit was

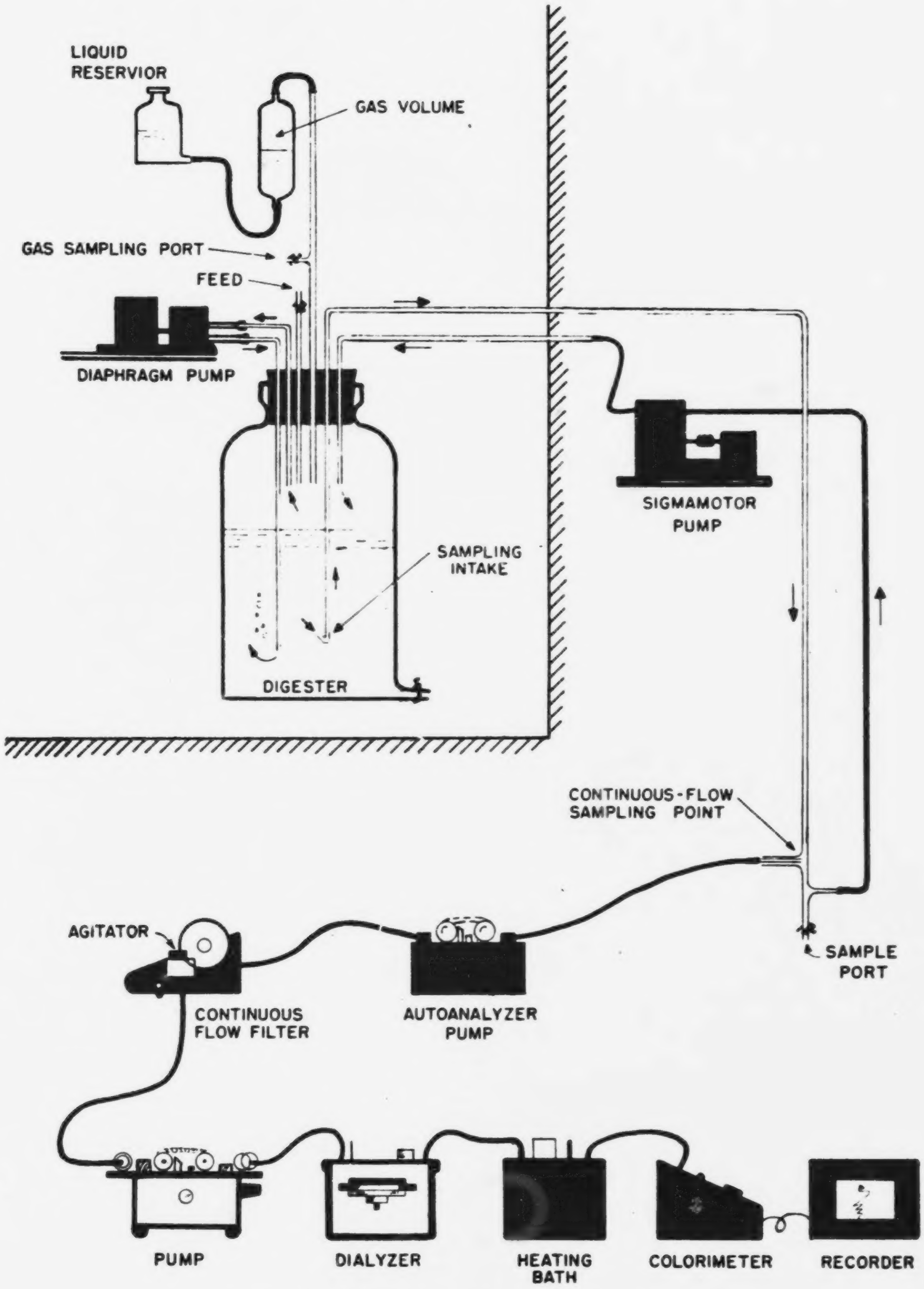

FIG. 2. *Digester continuous flow with enzymatic glucose monitoring.*

achieved by lowering a sampling tube from the Autoanalyzer directly into the aeration chamber (Fig. 3).

Enzymatic reactions. Glucose oxidase was first described by Muller (11). Its specificity for β-D-glucose was later shown by the work of Keilen and Hartree (7, 8). The reaction proceeding was as follows:

$$\text{glucose} + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

Keston (Proc. Meeting Am. Chem. Soc., 129th, p. 31 c) in 1956 described a "novel" idea for the simultaneous use of two enzyme systems for determining glucose by use of *o*-tolidine color change. Huggett and Nixon (4, 5, 6) in 1957 described this method for manual estimation of glucose (in blood) with use of glucose oxidase, horseradish peroxidase, and *o*-dianisidine. The hydrogen peroxide formed in the presence of peroxidase, giving chromogenic oxidation products, the intensity of whose color is proportional to the amount of color initially present. Hill and Kessler (3), as well as Discombe (2) and Saifer (12), adapted this method for the Autoanalyzer, the reaction being:

$$\text{glucose} + O_2 + \text{water} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 + o\text{-dianisidine} \xrightarrow{\text{horseradish proxidase}} \text{water} + \tfrac{1}{2}O_2 + \text{color} \quad (2)$$

The color formed is detected optimally at 460 mμ.

Temperature. Temperature affects the kinetics of reactions in biological systems. For the anaerobic experiments presented herein, digestion units were housed in a constant-temperature room maintained at a temperature of 35 C ± 0.1 C. This is the commonly employed temperature for such units, and can be considered to be typical of most field digestion treatment. Temperature in aerobic waste treatment, in contrast to anaerobic treatment, is conducted at the prevailing temperature of the waste water and varies with each season. For purposes of this experiment, however, the activated sludge units were held at room temperatures in the range of 23 ± 1 C. In both the aerobic and anaerobic studies, temperature

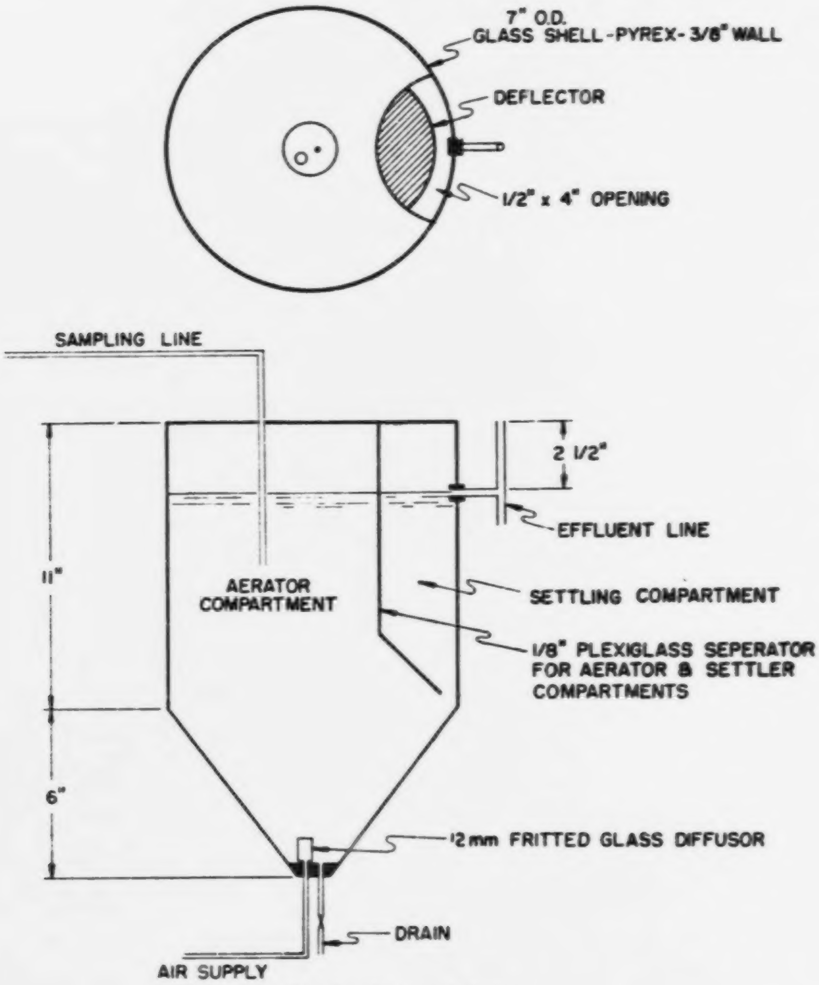

FIG. 3. *Activated sludge.*

was maintained constant and no effort was made to determine its effect.

Anaerobic digester. The anaerobic digester was generally fed an amount of glucose to give a concentration of 1,000 mg/liter. The evening prior to the run, the diaphragm pump was turned on to recirculate gases and agitate the digester. To prevent settling or stagnation in the sample line, the Sigmamotor pump was also allowed to recirculate liquid samples overnight past the continuous-flow sample port. These pumps operated continuously up to and during the course of the experiment.

The morning of the glucose run, a steady base line was obtained on the Autoanalyzer with distilled water and reagents; then glucose standards were run. Just prior to slug-feeding glucose, a sample was removed from the digester for determination of *p*H, volatile acids, alkalinity, COD, and gas composition. As soon as the standards had been detected, the sample line to the digester was opened to determine the concentration of glucose prior to feeding. At no time was glucose detected in the digester before initiating a run, and the base line obtained was identical to that obtained with distilled water. At time zero, a slug of glucose calculated at approximately 1,000 mg/liter was fed to the digester. During the course of the experiment, liquid samples were removed from the digester for analysis. Gas-volume measurements were also taken during the run and for a period of time following the experiment. When the glucose concentration in the digester had diminished to the level of the pre-existing base line and remained steady, the analyzer was allowed to continue sampling from the digester for another 20 min. This was followed by distilled-water sampling, a set of glucose standards, and distilled water again.

Activated sludge unit. On the day of the experiment, solids were wasted and nutrients were added as usual. However, prior to adding glucose, a sample line from the Autoanalyzer was lowered into the aeration chamber. Similarly, as with the anaerobic run, a base line was obtained which was identical to that of distilled water. At time zero, a slug of glucose was added and monitored continuously until glucose could no longer be detected. Glucose standards were run immediately before and after the run.

For both aerobic and anaerobic units, continuous glucose analysis showed no drift of base line, or significant variation in the values of standards before and after the run.

RESULTS

Anaerobic digester. Two continuous runs were made for the disappearance of glucose in anaerobic digesters by use of the enzyme procedure and the Autoanalyzer. Both glucose runs employed slugs of glucose of approximately 1,000 mg/liter. Buildup of glucose concentration to a maximum was rapid and was obtained in approximately 5 min for the two runs. After periods of 43 and 31 min, respectively, of continuous monitoring, no glucose could be detected in the digesters. No noticeable lag was noted following the addition of glucose, and disappearance was observed to be almost perfectly linear. A slight tailing, departing from linearity, was detected toward the end of both runs.

A plot of the data for glucose disappearance following the slug at time zero is shown for both runs as Fig. 4. The equations obtained for the disappearance of glucose are:

$$\text{Run 1, } C = C_0 - 1{,}300t$$

$$\text{Run 2, } C = C_0 - 1{,}490t$$

where t = time (hr), C = glucose concentration at any time t, and C_0 = initial concentration of glucose: run no. 1, 930 mg/liter; run no. 2, 760 mg/liter. The values for C_0 are obtained by extending the straight line portion of the curves shown in Fig. 4 to time zero. It should be noted

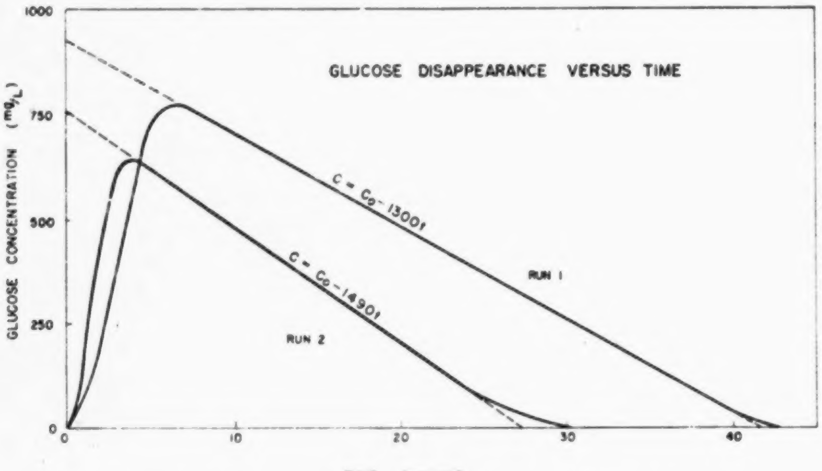

FIG. 4. *Glucose disappearance versus time* (AN*aerobic*).

that, although it was planned to have the same amount of glucose in each of the two runs, the initial concentration C_0 was different. The fact that the values of C_0 are not in close agreement with the glucose added makes an error in weighing a distinct possibility, although other reasons can be advanced. Future runs should resolve this question.

Activated sludge unit. Results from the slug addition of glucose to the activated-sludge unit are shown in Fig. 5. The concentration of glucose used was approximately 1,350 mg/liter. Maximal concentration was detected 5 min after addition and diminished linearly, as shown, until low concentrations (less than 30 mg/liter) were reached, at which time some tailing was noted. As with the anaerobic run, no lag was noted following the addition of the substrate.

The equation obtained was as follows:

$$C = C_0 - 388\, t$$

where t = time (hr), C = glucose concentration at any time t, and C_0 = initial concentration of glucose: 1,310 mg/liter.

DISCUSSION

Data for all the runs reported in these studies gave the same steady baseline with use of the glucose reagents for both distilled water and the biological unit contents prior to the run and following depletion of the added glucose. Considering the specificity of the enzyme detector system, it may reasonably be assumed that "true" glucose is being measured in the digester, and the possibility of other compounds causing similar peaks or reactions to occur is unlikely.

For the course of this experiment, it is also reasonably safe to assume that the bacterial numbers, as well as the enzyme in the system, will remain fairly constant, considering the long generation times characteristic for bacteria in anaerobic systems. In such systems, bacteria are known to reproduce at slow rates, reasonably beyond 1 hr, and certainly longer than the time required for the disappearance of glucose in these experiments. For the aerobic system, this argument may not be valid. Nevertheless, considering the linearity of the results and the low feed concentration, this factor does not appear to be significant.

According to currently held theories of enzyme reactions, and the accepted mathematical analysis of Michaelis-Menton (10), enzyme reactions take place by reversible, complex reactions involving the enzyme (*E*), substrate (*S*), enzyme-substrate complex (*ES*), and the end product (*P*). This is usually represented as follows:

$$(E) + (S) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES) \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (E) + (P) \qquad (1)$$

By this mathematical treatment, when equilibrium conditions are reached, and the equation rearranged, the Michaelis-Menton equation may be written as:

$$(ES) = \frac{(E)\,(S)}{(S) + \dfrac{k_2 + k_3}{k_1}} \qquad (2)$$

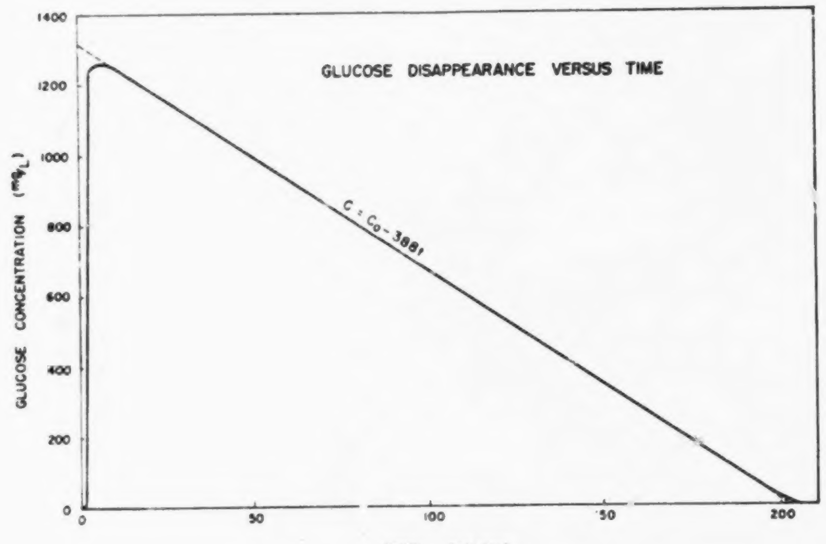

FIG. 5. *Glucose disappearance versus time ([illegible]aerobic).*

If the concentration of the substrate is very great in comparison to the rate constant ratio K_m (the Michaelis constant):

$$K_m = \frac{k_2 + k_3}{k_1} = \frac{(S)\,[(E) - (ES)]}{(ES)} \quad (3)$$

then the enzyme-substrate complex (ES) in equation 2 is seen to be independent of the substrate concentration (S) and the rate of formation and decomposition of the enzyme-substrate complex (ES) does not vary with concentration. Hence, the disappearance of the substrate is a zero-order reaction.

A graph of the data obtained (Fig. 4 and 5), with a plot of the calculated equations superimposed, shows concentration of substrate to be a function of time in a straight line relationship. These plots show the rate of reaction dc/dt to be constant, thus prescribing a zero-order reaction, which is consistent with the predicted behavior for enzymatic reactions of the Michaelis-Menton theory.

In aerobic waste-treatment systems, such as activated sludge or trickling filters, Wuhrmann, as well as others (1, 13–16), also demonstrated that concentrations of pure compounds in a suspension of microorganisms will decrease in concentration linearly with respect to time, and hence be zero-order reactions.

Inasmuch as even low substrate concentrations will apparently suffice for maximal saturation velocities of known substrate receptor sites, transport mechanisms for the substrate molecules to the receptor sites appear to be the only factor determining the kinetics of substrate disappearance in a medium. Since this mechanism is mostly diffusion, it follows that a concentration decrease should follow a zero-order reaction.

Following this line of reasoning, which was advanced by Wuhrmann (15), it would appear that as soon as the external substrate concentration has diminished to the point that diffusion rates become less than the rate of fixation to the cell receptors, a change in the reaction-rate characteristics will occur. These concentrations are considered to be low, even beyond the limits of detection employed in the laboratory. At low levels of substrate, it can be seen that tailing or departure from linearity might be expected. Analysis of our laboratory data, as evidenced by the gradual tailing observed in both runs, is consistent with this interpretation.

As a tool for the study of continuously operating treatment systems, such as the anaerobic digesters, the activated sludge process, or any unit operation in biological waste treatment, the principle of continuous monitoring for a substrate is a valuable approach and can yield much useful information. By this methodology it is possible to develop more precise mathematical descriptions relating to the kinetics involved in substrate disappearance, and hence, eventually, to provide more meaningful data relating to waste-treatment processes. Less direct, and perhaps more important, is the fact that continuous monitoring provides a method of study of the waste-treatment process which can enable an investigator to study one specific (or principal) reaction rate with respect to a wide number of variables.

ACKNOWLEDGMENT

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LITERATURE CITED

1. DAWSON, P. S. S., AND S. J. JENKINS. 1950. The oxygen requirements of activated sludge determined by manometric methods. II. Chemical factors affecting oxygen uptake. Sewage Ind. Wastes **22**:490–507.
2. DISCOMBE, G. 1963. An inexpensive method for the estimation of true glucose in blood and other fluids by the autoanalyzer. J. Clin. Pathol. **16**:170–172.
3. HILL, J. B., AND G. KESSLER. 1961. An automated determination of glucose utilizing a glucose-oxidase peroxidase system. J. Lab. Clin. Med. **57**:970–980.
4. HUGGETT, A. St. G., AND D. A. NIXON. 1957. Use of glucose oxidase, peroxidase, and o-dianisidine in determination of blood and urinary glucose. Lancet **2**:368–370.
5. HUGGETT, A. ST. G., AND D. A. NIXON. 1957. The determination of blood glucose using mixed enzyme-oxygen acceptor reagent. Biochem. J. **66**:12P.
6. HUGGETT, A. St. G., AND D. A. NIXON. 1957. The determination of blood glucose using mixed enzyme oxygen acceptor reagent. J. Physiol. **137**:3P.
7. KEILIN, D., AND E. F. HARTREE. 1952. Specificity of glucose oxidase (notatin). Biochem. J. **50**: 331–332.
8. KEILIN, D., AND E. F. HARTREE. 1948. Properties of glucose oxidase (notatin). Biochem. J. **42**:221–225.
9. LUDZACK, F. J. 1960. Activated sludge model. J. Water Pollution Control Federation **32**(**6**): 605–609.
10. MICHAELIS, L., AND M. L. MENTEN. 1913. Die Kinetik der Invertinwirkung. Biochem. Z. **49**:333–369.
11. MULLER, D. 1928. Studien uber ein neues Enzym Glykoseoxydase Biochem. Z. **199**:136–139.
12. ROBIN, M., AND A. SAIFER. 1965. Determination

of glucose in biologic fluids with an automated enzymatic procedure. Clin. Chem. **11**:840–845.

13. Water Pollution Research Laboratory. 1955. Annual Report, p. 51–57. Her Majesty's Stationery Office, London.
14. Winzler, R. J. 1941. The respiration of baker's yeast at low oxygen tension. J. Cellular Comp. Physiol. **17**:263–267.
15. Wuhrmann, K. 1963. Discussion, p. 287. *In* W. W. Eckenfelder and B. J. McCabe [ed.], Advances in biological waste treatment. Pergamon Press, New York.
16. Wuhrmann, K. 1956. Factors affecting efficiency and solids production in the activated sludge process, p. 49–65. *In* B. J. McCabe and W. W. Eckenfelder [ed.], Biological treatment of sewage and industrial wastes, vol. 1. Reinhold Publishing Corp., New York.

APPENDIX IV

SAMPLE CALCULATIONS

A. Radioisotope Studies

A series of sample calculations will be presented in this section. These calculations were made based on the data collected. Their use was necessary to determine the efficiency of the substrate reduction, the specific activity of the methane and carbon dioxide produced, and other pertinent information.

The calculations were divided into two general categories based on the isotope fed and the manner in which the isotopes were fed. In the first category were the calculations in which the isotopes were fed daily for more than 30 days (long term) while the second category included the calculations in which the radiochemicals were fed only on one day. The following are the calculations of two typical runs which represent each of the two categories.

1. Long term isotope use

The study was conducted using L(+)-Glutamic Acid as the substrate to which was added DL-Glutamic Acid-2C-14.

a. Gas production during 24 hrs. = 845 ml

b. Volume of gas in the digester

The gas reading on the 1300 ml glass gas collection cylinder was 1150 ml. The estimated gas volume for the portion above the zero reading in the 1300 ml cylinder including the space above the sludge in the digester, the tygon tubing connecting the digester and the 1300 ml cylinder was 905 ml. The total volume of gas was 1150 + 905 = 2055 ml.

c. Gas obtained from boiled sludge

The amount of sludge used for boiling was 255 ml and the gas volume obtained from boiling this sludge was 732 ml at 25°C (298°K). The total sludge volume in the digester was 1500 ml. Therefore, the gas volume that would be obtained from 1500 ml of digester liquor at 35°C (308°K) would be:

$$732 \times \frac{1500}{255} \times \frac{308}{298} = 732 \times \frac{1500}{255} \times 1.031 = 4444 \text{ ml}$$

d. Total gas

$2055 + 4444 = 6499$ ml

e. Amount of CH_4

The Handbook of Chemistry (44) gives the solubility of CH_4 at 1 atmosphere CH_4 and 35°C as 0.00173 grams per 100 ml water. According to Charles Law, the volume of CH_4 gas in 1500 ml water at 35°C (308°K) and 1 atm. CH_4 can be computed:

$$CH_4 \text{ in 1500 ml } H_2O = \frac{(1500)(0.00173)(22400)(308)}{(100)(16)(273)} = 41 \text{ ml}$$

In an atmosphere of 62.5% CH_4 the amount of CH_4 theoretically soluble would be 41 X 0.625 = 25.6 ml.

The CH_4 actually soluble amounted to 4444 X 0.0098 = 43.6 ml. The gaseous CH_4 was 2055 X 0.625 = 1284 ml. The total CH_4 then was 43.6 + 1284 = 1328 ml.

f. Amount of CO_2

The soluble CO_2 was 4444 X 0.875 = 3890 ml, while the gaseous CO_2 was 2055 X 0.361 = 741 ml. The total CO_2 was 4631 ml.

g. Check total gas

The total CH_4 and CO_2 was 1328 + 4631 = 5959 ml. The nitrogen volume was 2055 X 0.0136 = 28 ml while the soluble nitrogen was 4444 X 0.115 = 511 ml. The total volume of CO_2, CH_4 and nitrogen then was 5959 + 28 + 511 = 6498 ml which is quite close to the volume obtained in section d, so the computation checks within experimental error.

h. Gas production during run

The CH_4 at the end of the run was 2055 X 0.625 = 1284 ml while at the start of the run it was (2055-845) X 0.598 = 723 ml. So the CH_4 production during the run was 1284 - 723 = 561 ml.

The CO_2 at the end of the run was 2055 X 0.361 = 741 ml, while at the start of the run it was (2055-845) X 0.372 = 450 ml. So the gaseous CO_2 production during the run was 741 - 450 = 291 ml.

In the Handbook of Chemistry and Physics (45) the solubility of CO_2 at 1 atm. CO_2 and 35°C is reported as 0.1105 gm per 100 ml water. Using the factors previously outlined in section e, the ml of CO_2 gas in solution at 35°C and 1 atm. CO_2 is computed:

$$CO_2 \text{ in 100 ml } H_2O = \frac{(0.1105)(22400)(308)}{(44)(273)} = 63.4 \text{ ml}$$

At 36.1% CO_2, the soluble CO_2 in 100 ml feed was 63.4 X 0.361 = 23 ml. The total CO_2 production during the run would be 291 + 23 = 314 ml.

i. Gaseous CO_2 and CH_4 produced during the run

561 + 291 = 852 ml

j. Percent COD reduction on chemical basis

The percent COD reduction was calculated by use of the following formula:

$$\text{\% COD Reduction} = \frac{\text{Ave COD fed daily} - \left[\begin{matrix}\text{Ave COD remaining}\\ \text{in mixed effluent}\end{matrix}\right]}{\text{Ave COD fed daily}} \times 100$$

All values of COD were expressed in mg/l.

The COD fed daily was 1400 mg per 100 ml feed sample and the COD remaining in the mixed effluent was 691 mg/l. Therefore, the % COD Reduction is calculated:

$$\frac{(1400)(10) - 691}{(1400)(10)} \times 100\% = 95.0\%$$

k. Percent COD reduction on CH_4 basis

This calculation is based on the COD equivalent of methane, which may be readily computed from the following chemical equation:

$$CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O$$

Total weight(gm) 16   32X2=64

1 mole CH_4 is equivalent to 64 grams of COD

1/64 mole CH_4 is equivalent to 1 gram of COD

At 35°C and 1 atmosphere pressure, 1/64 mole of gas occupies:

$$\frac{308}{273} \times 22400 \times \frac{1}{64} = 395 \text{ ml.}$$

Therefore 395 ml CH_4 is equivalent to 1 gram COD at 35°C and 1 atmosphere pressure.

The following formula can then be derived:

$$\begin{array}{l}\text{\% COD Reduction} \\ \text{on } CH_4 \text{ basis}\end{array} = \frac{(\%CH_4 \text{ in gas})(\text{Ave Gas Produced in ml})}{(395 \text{ ml } CH_4/\text{gm COD fed})(\text{gm COD fed})}$$

From the data shown in the Appendix Tables the % COD Reduction based on CH_4 is calculated:

$$\frac{845 \times 0.625}{395 \times 1.4} \times 100 = 95.4\ \%$$

l. Total activity

$$CO_2 - 524 \times \frac{4631}{610} = 3980 \text{ counts/minute}$$

$$CH_4 - 4313 \times \frac{1328}{625} = 9160 \text{ counts/minute}$$

m. Specific activity

$$CO_2 = \frac{524}{610} = 0.86 \text{ counts/min/ml}$$

$$CH_4 = \frac{4313}{625} = 6.91 \text{ counts/min/ml}$$

$$\text{Specific Activity } \frac{CO_2}{CH_4} = \frac{0.86}{6.91} = 0.124$$

n. Effects of addition of Na^+ and HCl on CO_2 production

In order to maintain a neutral pH and a reasonable alkalinity, either sodium hydroxide or hydrochloric acid was added to the digesters daily. The following computations will show the effects of addition of Na^+OH and HCl on CO_2 production.

(1) Effect of NaOH addition

$$Na^+ + OH^- + CO_2 \longrightarrow NaHCO_3$$

$$\frac{\begin{array}{ll} 23\text{ g} & 22.4\text{ liters} \\ 100\text{ mg} & (x)\text{ ml} \end{array}}{x = \frac{100 \text{ X } 22.4}{23} = 97.4\text{ ml}}$$

at 35°C - $97.4 \text{ X } \frac{308}{273} = 109.7$ ml

Therefore, 100 mg Na^+ absorbs 109.7 ml CO_2 at 35°C and 1 atmosphere pressure.

(2) Effect of HCl addition

$$HCl + NaHCO_3 \longrightarrow NaCl + H_2O + CO_2$$

$$\frac{\begin{array}{ll} 36.46\text{ g} & 22.4\text{ liters} \\ 100\text{ mg} & (x)\text{ ml} \end{array}}{x = \frac{100 \text{ X } 22.4}{36.46} = 61.4\text{ ml}}$$

at 35°C - $61.4 \text{ X } \frac{308}{273} = 69.3$ ml

Therefore, 100 mg HCl releases 69.3 ml CO_2 at 35°C and 1 atmosphere pressure.

Since 900 mg of hydrochloric acid and 560 mg of Na^+ were fed daily to each of the L(+)-Glutamic Acid units, the CO_2 production during run was affected:

$$69.3 \text{ X } \frac{900}{100} - 109.7 \text{ X } \frac{560}{100} = 9.2 \text{ ml}$$

That is, 9.2 ml of CO_2 was released. When comparing this to the total CO_2 production it is a negligible quantity and was not included in the calculations.

2. One day slug feed of isotope

Using this method of study there were only slight modifications in the calculations of the activity of the gases produced when using $NaHC^{14}O_3$ or labeled compounds which yield labeled methane primarily from carbon dioxide reduction. Only the modifications are outlined as follows:

Activity of the gas produced

Total CO_2 at end of run = 4731 ml

CO_2 production during run = $\underline{422.5 ml}$

Total CO_2 at start of run = 4308.5 ml

CH_4 production during run = 543 ml

Total radioactivity CO_2 at end of run = 33300 counts/minute

Total radioactivity CH_4 at end of run = 452 counts/minute

Specific Activity of CO_2 at start of run $\frac{33752}{4308.5} = 7.84$

Specific Activity of CO_2 at end of run = $\frac{33300}{4731} = 7.05$

Average specific activity of $CO_2 = \frac{7.84 + 7.05}{2} = 7.45$

Specific Activity of $CH_4 = \frac{452}{543} = 0.833$

Specific Activity $\frac{CO_2}{CH_4} = \frac{7.45}{0.833} = 8.94$

B. Warburg Studies

The following are the calculations for the Warburg analysis of a unit which is considered to be typical. The calculations shown are based on the study using butyric acid as the substrate to which was added the sludge liquor of glutamic acid digester #103. The data collected in this study are given in the Appendix tables.

(a) Correction for Gas Production

The gas production for the two unfed controls were 2.2 and 4.0 ml or an average value of 3.1 ml.

The change of the solubility of carbon dioxide in the 53 ml sample was estimated. The quantity of CO_2 gas in solution at 35°C and 1 atm. CO_2 was computed using the method previously outlined to be 63.4 ml CO_2 per 100 ml H_2O. The soluble CO_2 is therefore, equal to the percent CO_2 in the gaseous phase, expressed as a fraction, multiplied by 63.4 ml.

$$\% \; CO_2 \text{ in flask at start of run} = 31.5\%$$

$$\text{ml } CO_2 \text{ in 53 ml sample at start} = 63.4 \times \frac{53}{100} \times 0.315$$

$$= 10.6 \text{ ml}$$

The theoretical percentage of carbon dioxide in the gas produced from butyric acid degradation may be readily computed from the following chemical equations:

$$CH_3CH_2CH_2COOH + NaOH \longrightarrow CH_3CH_2CH_2COONa + H_2O$$

$$CH_3CH_2CH_2COONa + 2H_2O \longrightarrow NaHCO_3 + 1/2CO_2 + 5/2CH_4$$

$$\% \; CO_2 = \frac{1/2(100)}{1/2 + 5/2} = 16.7\%$$

The volume above the liquor surface in the flask is approximately 80 ml. The gas produced during the run was 6.1 ml. The percentage of carbon dioxide in the gas and liquid phases of the flask at the completion of the test was estimated as follows:

$$\frac{(80-6.1) \times 0.315 + 6.1 \times 0.167}{80} \times 100 = 30.4\%$$

$$\text{ml } CO_2 \text{ in 53 ml sample} = 63.4 \times \frac{53}{100} \times 0.304 = 10.2 \text{ ml}$$

Therefore, 10.6 - 10.2 = 0.4 ml of CO_2 evolved from the sludge sample during the test.

Then, the corrected gas production can be computed:

$$6.1 - 3.1 - 0.4 = 2.6 \text{ ml}$$

Although the correction for carbon dioxide solubility was very small and could have been considered negligible in this case, for other substrates, it amounted to a significant correction.

(b) Percent Soluble COD Reduction

The percent COD reduction was calculated by use of the following formula:

$$\% \text{ COD Reduction} = \frac{a+b-c}{a} (100)$$

in which a = COD of the feed in mg/l

b = Initial soluble COD of the sludge liquor in mg/l

c = Soluble COD of the mixed sample at the completion of the test in mg/l

Therefore, from the data collected during the Warburg run the COD reduction was calculated:

% COD Reduction = 29.6%

(c) Theoretical Gas Production for Complete Destruction of Substrate

To this unit, 43.7 mg COD of butyric acid was added. From the chemical equations previously presented in calculation (a), it was shown that three moles of gas were produced for each mole of butyric acid degraded.

The theoretical gas production for complete destruction of butyric acid may be readily computed from the following chemical equation:

$$CH_3CH_2CH_2COOH + 5O_2 \longrightarrow 4CO_2 + 4H_2O$$

88 160

Total Weight (gm)

x 43.7 mg

$$x = 24.00 \text{ mg or } \frac{24 \times 10^{-3}}{88} = 0.273 \times 10^{-3} \text{ mole}$$

Therefore, 24.0 mg or 0.273×10^{-3} moles of butyric acid is equivalent to 43.7 mg of COD. The ml of theoretical gas production at 35°C at 1 atm. is computed: (for 3 moles)

$$3 \times 0.273 \times 10^{-3} \times 22400 \times \frac{308}{273} = 20.6 \text{ ml}$$

APPENDIX

V TYPICAL ANAEROBIC DIGESTER DATA

TABLE 1

LACTIC ACID (1-C^{14}Lactate) DATA-DIGESTER #105

October 16, 1967 - Slug fed 1-C^{14}-Lactate

Daily feed: 1400 mg COD of Lactic Acid
150 mg Na^+
2 ml of Nutrient 4A
2 ml of Nutrient M

Date	Time	Daily Gas ml	pH	Alk. mg/l as $CaCO_3$	V.A. mg/l as HAc	Mix Effl. COD mg/l	% of Gas Composition N_2	CH_4	CO_2
10/13	11AM	640	7.3	3300	86	893	2.0	66.8	31.1
10/14	11AM	768	7.3	3350	86	967	2.2	65.5	32.2
10/15	11AM	840	7.3	3300	70	880	2.4	65.5	32.0
10/16	11AM	720	7.3	3300	52	843	2.4	65.0	32.5
10/17	11AM	680	7.3	3300	70	920	2.5	64.4	33.0

Sludge used for boiling = 395 ml

Gas obtained from boiled sludge = 690 ml

% Composition of the gas obtained from boiled sludge:

N_2 = 0.7 CH_4 = 0.3 CO_2 = 98.8

Total volume of gas in system at end of run = 4650 ml

Total CH_4 in system at end of run = 1260 ml

Total CO_2 in system at end of run = 3313 ml

CH_4 produced during run = 429 ml

CO_2 produced during run = 416 ml

COD reduction on chemical basis = 93.4%

COD reduction on CH_4 basis = 79.3%

CO_2 absorbed in PEA solution at 35°C = 651 ml

CO_2 (CH_4 fraction) absorbed in PEA solution at 35°C = 661 ml

Counts per minute: (In PEA solution)

CO_2 fraction: 725000

CH_4 fraction: 99860

Total Activity of CO_2 = 3695000 counts/min

Total Activity of CH_4 = 191000 counts/min

Average Specific Activity of CO_2 = 1228 counts/min/ml

Average Specific Activity of CH_4 = 444 counts/min/ml

Average Specific Activity of Carbon Dioxide

$$\text{SA } CO_2 \text{ start of run} = \frac{3695000 + 191000}{3313 - 416} = 1342$$

$$\text{SA } CO_2 \text{ end of run} = \frac{3695000}{3313} = 1114$$

$$\text{Ave SA } CO_2 = \frac{1342 + 1114}{2} = 1228$$

Specific Activity of Methane Produced

$$\text{SA } CH_4 = \frac{191000}{429} = 444$$

$$\text{Specific Activity } \frac{CO_2}{CH_4} = 2.77$$

TABLE 2

LACTIC ACID ($NaHC^{14}O_3$) DATA-DIGESTER #102

January 30, 1967 - Slug fed $NaHC^{14}O_3$

Daily feed: 1400 mg COD of Lactic Acid
150 mg Na^+
2 ml of Nutrient 4A
2 ml of Nutrient M

Date	Time	Daily Gas ml	pH	Alk. mg/l as $CaCO_3$	V.A. mg/l as HAc	Mix Effl. COD mg/l	% of Gas Composition N_2	CH_4	CO_2
1/27	Noon	780	7.2	3150	171	783	1.6	65.5	32.8
1/28	9AM	770	7.2	3100	181	788	1.6	64.4	32.9
1/29	Noon	710	7.2	3100	226	786	1.8	65.0	33.1
1/30	Noon	710	7.2	3100	137	776	1.7	65.9	32.3
1/31	Noon	695	7.1	3100	223	767	1.8	65.8	32.3

Sludge used for boiling = 340 ml

Gas obtained from boiled sludge = 595 ml

% Composition of the gas obtained from boiled sludge:

N_2 = 0.8 O_2 = 0.2 CH_4 = 0.3 CO_2 = 98.7

CO_2 absorbed in PEA solution at 35°C = 627 ml

CO_2 (CH_4 fraction) absorbed in PEA solution at 35°C = 635 ml

Total volume of gas in system at end of run = 4733 ml

Total CH_4 in system at end of run = 1339 ml

Total CO_2 in system at end of run = 3329 ml

CH_4 produced during run = 456 ml

CO_2 produced during run = 410 ml

COD reduction on chemical basis = 94.5%

COD reduction on CH_4 basis = 83%

CO_2 absorbed in PEA solution at 35°C = 627 ml

CO_2 (CH_4 fraction) absorbed in PEA solution at 35°C = 635 ml

Counts per minute: (In PEA solution)

CO_2 fraction: 6898
CH_4 fraction: 918

Total Activity of CO_2 = 36600 counts/min

Total Activity of CH_4 = 1935 counts/min

Average Specific Activity of CO_2^* = 12.10 counts/min/ml

Average Specific Activity of CH_4^* = 4.24 counts/min/ml

Specific Activity $\frac{CO_2}{CH_4}$ = 2.85

* See Sample Calculations and Table 1 Appendix V

TABLE 3

L(+)-GLUTAMIC ACID($NaHC^{14}O_3$) DATA - DIGESTER #103

September 5, 1967 - Slug fed $NaHC^{14}O_3$

Daily feed: 1400 mg COD of L(+)-Glutamic Acid
900 mg of HCl; 560 mg of Na^+
2 ml of Nutrient 4A
2 ml of Nutrient M

Date	Time	Daily Gas ml	pH	Alk. mg/l as $CaCO_3$	V.A. mg/l as HAc	Mixed Effl. COD mg/l	% of Gas Composition N_2	CH_4	CO_2
9/3	Noon	885	7.3	4100	86	504	0.84	56.46	42.7
9/4	11AM	900	7.3	4100	86	512	0.92	56.78	42.3
9/5	10AM	875	7.3	4200	120	533	1.0	57.4	41.6
9/6	10AM	950	7.2	4250	103	580	1.0	57.3	41.7

Sludge used for boiling = 325 ml

Gas obtained from boiled sludge = 815 ml

% Composition of the gas obtained from boiled sludge:

N_2 = 0.8 CH_4 = 0.2 CO_2 = 99.0

CO_2 absorbed in PEA solution at 35°C = 624 ml

CO_2 (CH_4 fraction) absorbed in PEA solution at 35°C = 625 ml

Total volume of gas in system at end of run = 6020 ml

Total CH_4 in system at end of run = 1234 ml

Total CO_2 in system at end of run = 4731 ml

CH_4 produced during run = 543 ml

CO_2 produced during run = 423 ml

COD reduction on chemical basis = 96.0%

COD reduction on CH_4 basis = 98.5%

Counts per minute: (In PEA solution)

CO_2 fraction: 4402

CH_4 fraction: 229

Total Activity of CO_2 = 33300 counts/min

Total Activity of CH_4 = 452 counts/min

Average Specific Activity of CO_2^* = 7.45 counts/min/ml

Specific Activity of CH_4^* = 0.833 counts/min/ml

Specific Activity $\frac{CO_2}{CH_4} = 8.94$

* See Sample Calculations Appendix IV and Table 1 Appendix V.

TABLE 4

L(+)-GLUTAMIC ACID (2C^{14}DL-GLUTAMIC ACID) DATA - DIGESTER #100

Daily Feed: 1400 mg COD of L(+)-Glutamic Acid
DL - glutamic acid-2C-14
900 mg of HCl, 560 mg of Na$^+$
2 ml of Nutrient 4A
2 ml of Nutrient M

Date	Time	Daily Gas ml	pH	Alk. mg/l as $CaCO_3$	V.A. mg/l as HAc	Mixed Effl. COD mg/l	% of Gas Composition		
							N_2	CH_4	CO_2
12/1/67	11AM	850	7.4	4700	771	-	-	-	-
12/2/67	11AM	910	7.3	4800	514	705	0.69	60.9	38.4
12/3/67	11AM	850	7.3	4850	274	717	0.90	60.0	39.1
12/4/67	9AM	710	7.4	4700	411	705	2.93	59.8	37.2
12/5/67	11AM	845	7.3	4950	171	691	1.36	62.5	36.1

Sludge used for boiling = 255 ml

Gas obtained from boiled sludge = 732 ml

% Composition of the gas obtained from boiled sludge:

N_2 = 11.5 CH_4 = 0.98 CO_2 = 87.5

CO_2 absorbed in PEA solution at 35°C = 610 ml

CO_2 (CH_4 fraction) absorbed in PEA solution at 35°C = 625 ml

Total volume of gas in system at end of run = 6499 ml

Total CH_4 in system at end of run = 1328 ml

Total CO_2 in system at end of run = 4631 ml

CH_4 produced during run = 561 ml

CO_2 produced during run = 314 ml

COD reduction on chemical basis = 95.0%

COD reduction on CH_4 basis = 95.4%

Counts per minute: (In PEA solution)

CO_2 fraction: 524
CH_4 fraction: 4313

Total Activity of CO_2 = 3980 counts/min

Total Activity of CH_4 = 9160 counts/min

Specific Activity of CO_2^* = 0.86 counts/min/ml

Specific Activity of CH_4^* = 6.91 counts/min/ml

Specific Activity $\frac{CO_2}{CH_4} = 0.124$

* See Sample Calculations Appendix IV

APPENDIX

VI TYPICAL WARBURG DATA

TABLE 1

RESULTS OF WARBURG RUN 4 - SUCCINIC ACID
DIGESTER #106

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6)%
3	Succinic Acid	22.4	10.2	78	8.3	123
4	Succinic Acid	27.4	15.2	92	8.3	183
5	Propionic Acid	4.7	10.9	98	23.8	45
6	Propionic Acid	8.6	14.8	93	23.8	62
7	Lactic Acid	4.7	10.9	30	28.3	38
8	Lactic Acid	6.7	12.9	10	28.3	45
9	Methylmalonic Acid	6.3	12.5	65	42.3	29
10	Methylmalonic Acid	6.0	12.2	69	42.3	28
11	Acetic Acid	23.0	29.2	90	28.2	103
12	Acetic Acid	2.0	8.2	103	28.2	29
13	2-Ketobutyric Acid	11.0	17.2	28	31.7	54
14	2-Ketobutyric Acid	9.6	15.8	20	31.7	49
15	3-hydroxy-propionic Acid	3.5	9.7	6	31.6	30
16	3-hydroxy-propionic Acid	3.8	10.0	-	31.6	31
17	2-Ketoglutaric Acid	3.9	10.1	13	35.2	29
18	Control - Endogenous	3.4	9.6	-	-	-

* Corrected for Na^+ and/or acid added.

TABLE 2

RESULTS OF WARBURG RUN 5 - LACTIC ACID

DIGESTER #12

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6) %
3	Endogenous	3.1	2.8	63.7	-	-
4	Endogenous	2.5	(ave)	75.0	-	-
5	Lactic Acid	42.2	35.4	106.0	34.1	104
6	Lactic Acid	43.1	35.9	105.0	34.1	105
7	Pyruvic Acid	25.6	25.6	108.0	36.7	Leak[1]
8	Pyruvic Acid	38.0	40.0	97.7	36.7	109
10	Ethyl Alcohol	27.3	24.2	100	21.1	114.0
11	Sodium Formate	15.9	10.0	103.0	0	∞
12	Sodium Formate	19.0	12.5	104.9	0	∞
13	Acetic Acid	19.10	17.7	104.0	33.6	Leak[1]
14	Acetic Acid	39.10	33.6	102.9	33.6	100
15	Propionic Acid	29.30	27.7	105.3	27.9	99.8
16	Propionic Acid	36.60	35.3	106.3	27.9	126.0
17	Sodium Succinate	10.95	6.75	50.5	22.4	30.0
18	Sodium Succinate	7.8	9.0	38.8	22.4	40.4

* Corrected for Na^+ and/or acid added.

[1] Leak occurred during run and results not valid.

TABLE 3

RESULTS OF WARBURG RUN 6 - LACTIC ACID

DIGESTER #105

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6) %
3	Endogenous	5.6				
4	Endogenous	6.0				
5	Lactic Acid	17.38	18.58	98.3	39.6	Leak
6	Lactic Acid	32.20	32.40	99.0	39.6	82.0
7	Ethyl Alcohol	17.70	17.90	92.8	19.4	92.2
8	Ethyl Alcohol	21.10	21.30	117.0	19.4	108.0
9	α-Ketoglutarate	31.20	30.90	73.7	45.0	69.5
10	α-Ketoglutarate	38.30	38.50	90.7	45.0	85.7
11	Glyceraldehyde	37.30	37.50	79.0	50.4	74.5
12	Glyceraldehyde	42.40	42.60	89.0	50.4	84.4
13	Malonate	23.80	22.0	39.0	54.8	40.2
14	Malonate	26.40	25.10	45.2	54.8	45.8
15	Butyrate	33.90	33.60	133.5	29.3	114.2
16	Butyrate	38.90	39.10	140.5	29.3	134.3
17	Acetaldehyde	13.70	15.40	126.0	15.4	100.0
18	Acetaldehyde	11.40	12.85	105.0	15.4	84.0

* Corrected for Na^+ and/or acid added.

TABLE 4

RESULTS OF WARBURG RUN 7 - LACTIC ACID

DIGESTER #103

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6) %
3	α-Ketoglutarate	26.2	25.9	-	43.2	60.0
4	α-Ketobutarate	3.1	0	100	43.2	0
5	α-Ketobutarate	6.5	0	92.6	38.2	0
6	Propanol	5.3	-	18.2	38.2	0
7	Propanol	5.1	-	0.9	38.0	0
8	Endogeneous	2.4	2.0	0.97	-	-
9	Glyceraldehyde	24.9	25.2	63.3	36.0	70.0
10	Glyceraldehyde	29.2	29.8	72.2	36.0	83.7
11	Malonate	22.3	17.1	5.53	53.1	32.2
12	Malonate	12.4	5.6	-	53.1	10.5
13	Lactate	38.7	40.2	93.6	33.4	120
14	Lactate	21.2	21.1	90.4	33.4	63.2
15	Propionate	24.9	24.1	97.2	27.2	88.7
16	Propionate	17.9	16.7	-	27.2	61.5
17	Succinate	6.0	3.3	1.4	11.6	28.4
18	Succinate	4.0	1.5	1.0	11.6	12.9

* Corrected for Na^+ and/or acid added.

TABLE 5

RESULTS OF WARBURG RUN 8 - L(+)-GLUTAMIC ACID

DIGESTER #103

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6) %
3	Control	2.2				
4	Control	4.0				
5	L(+)-Glutamic Acid	31.4	28.3	80.8	33.1	85.5
7	NaButyrate	6.1	2.6	29.6	20.6	12.6
8	NaButyrate	8.4	4.8	18.8	20.6	23.2
9	Sodium Formate	14.1	9.2	45.9	0.00	∞
11	Pyruvic Acid	26.9	24.5	83.0	29.9	82.2
12	Pyruvic Acid	31.6	29.3	62.5	29.9	98.0
13	α-Ketoglutaric Acid	6.8	4.2	20.0	36.7	11.3
17	Sodium Acetate	32.2	24.9	96.5	17.7	141.0
18	Sodium Acetate	21.7	15.8	85.4	17.7	89.3

* Corrected for Na^+ and/or acid added.

TABLE 6

RESULTS OF WARBURG RUN 9 - L(+)-GLUTAMIC ACID

DIGESTER #6

(1) Unit No.	(2) Substrate	(3) Gas Prod. (ml)	(4) Corr. Gas Prod. (ml)	(5) % Soluble COD Reduction	(6) Theo. Gas Prod. for 100% COD Reduction*	(7) (4)/(6) %
3	Control	0.0				
4	Control	0.0				
5	L(+)-Glutamic Acid	30.1	30.1	59.4	31.8	94.6
7	Sodium Formate	13.3	11.2	78.2	0.0	∞
8	Sodium Acetate	31.6	26.5	66.4	17.8	149.0
9	Sodium Propionate	10.6	9.5	72.5	20.4	46.5
10	Sodium Propionate	34.9	31.1	-	20.4	152.4
11	NaButyrate	14.8	13.3	27.4	19.4	68.5
13	Valeric Acid	0.0	0.0	7.0	20.9	0.0
15	α-Ketoglutaric Acid	22.5	23.5	61.3	35.9	65.5
16	α-Ketoglutaric Acid	42.1	43.6	-	35.9	121.0
17	Mesaconic Acid	40.3	41.0	101.2	30.9	132.7
18	Mesaconic Acid	38.0	38.6	63.5	30.9	125.0

* Corrected for Na^+ and/or acid added.